

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
13 February 2003 (13.02.2003)

PCT

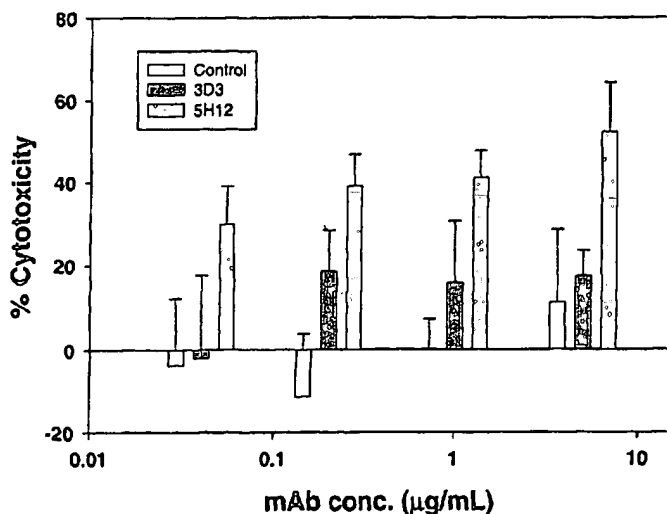
(10) International Publication Number  
WO 03/011878 A2

- (51) International Patent Classification<sup>7</sup>: C07H #262, CH-8046 Zurich (CH). BAILEY, James, E. (deceased).
- (21) International Application Number: PCT/US02/24739
- (22) International Filing Date: 5 August 2002 (05.08.2002)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data: 60/309,516 3 August 2001 (03.08.2001) US
- (71) Applicants: GLYCART BIOTECHNOLOGY AG [CH/CH]; Einsteinstrasse, CH-8093 Zurich (CH). BAILEY, Sean (legal representative of the deceased inventor) [US/US]; 450 N. Sycamore Avenue, No. 21, Los Angeles, CA 90036 (US).
- (72) Inventors: JEAN-MAIRET, Joel; Scheuchzerstrasse 94, CH-8006 Zurich (CH). UMAÑA, Pablo; Käferholzstrasse
- (74) Agents: COVERT, John et al.; Sterne, Kessler, Goldstein & Fox P.L.L.C., Suite 600, 1100 New York Avenue, Washington, DC 20005-3934 (US).
- (81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZM, ZW.
- (84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

[Continued on next page]

(54) Title: ANTIBODY GLYCOSYLATION VARIANTS HAVING INCREASED ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY

*In vitro* ADCC activity of new engineered chG250 mAb



(57) Abstract: The present invention relates to the field of glycosylation engineering of proteins. More particularly, the present invention relates to glycosylation engineering to generate proteins with improved therapeutic properties, including antibodies with increased antibody-dependent cellular cytotoxicity.

WO 03/011878 A2

WO 03/011878 A2



**Published:**

— without international search report and to be republished  
upon receipt of that report

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

ANTIBODY GLYCOSYLATION VARIANTS HAVING INCREASED  
ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY

BACKGROUND OF THE INVENTION

Field of the Invention

- [0001] The present invention relates to the field of glycosylation engineering of proteins. More particularly, the present invention relates to glycosylation engineering to generate proteins with improved therapeutic properties, including antibodies with increased antibody-dependent cellular cytotoxicity.

Background Art

- [0002] Glycoproteins mediate many essential functions in human beings, other eukaryotic organisms, and some prokaryotes, including catalysis, signaling, cell-cell communication, and molecular recognition and association. They make up the majority of non-cytosolic proteins in eukaryotic organisms. (Lis *et al.*, *Eur. J. Biochem.* 218:1-27 (1993)). Many glycoproteins have been exploited for therapeutic purposes, and during the last two decades, recombinant versions of naturally-occurring, secreted glycoproteins have been a major product of the biotechnology industry. Examples include erythropoietin (EPO), therapeutic monoclonal antibodies (therapeutic mAbs), tissue plasminogen activator (tPA), interferon- $\beta$ , (IFN- $\beta$ ), granulocyte-macrophage colony stimulating factor (GM-CSF), and human chorionic gonadotrophin (hCG). (Cumming *et al.*, *Glycobiology* 1:115-130 (1991)).
- [0003] The oligosaccharide component can significantly affect properties relevant to the efficacy of a therapeutic glycoprotein, including physical stability, resistance to protease attack, interactions with the immune system,

pharmacokinetics, and specific biological activity. Such properties may depend not only on the presence or absence, but also on the specific structures, of oligosaccharides. Some generalizations between oligosaccharide structure and glycoprotein function can be made. For example, certain oligosaccharide structures mediate rapid clearance of the glycoprotein from the bloodstream through interactions with specific carbohydrate binding proteins, while others can be bound by antibodies and trigger undesired immune reactions. (Jenkins *et al.*, *Nature Biotechnol.* 14:975-81 (1996)).

[0004] Mammalian cells are the preferred hosts for production of therapeutic glycoproteins, due to their capability to glycosylate proteins in the most compatible form for human application. (Cumming *et al.*, *Glycobiology* 1:115-30 (1991); Jenkins *et al.*, *Nature Biotechnol.* 14:975-81 (1996)). Bacteria very rarely glycosylate proteins, and like other types of common hosts, such as yeasts, filamentous fungi, insect and plant cells, yield glycosylation patterns associated with rapid clearance from the bloodstream, undesirable immune interactions, and in some specific cases, reduced biological activity. Among mammalian cells, Chinese hamster ovary (CHO) cells have been most commonly used during the last two decades. In addition to giving suitable glycosylation patterns, these cells allow consistent generation of genetically stable, highly productive clonal cell lines. They can be cultured to high densities in simple bioreactors using serum-free media, and permit the development of safe and reproducible bioprocesses. Other commonly used animal cells include baby hamster kidney (BHK) cells, NS0- and SP2/0-mouse myeloma cells. More recently, production from transgenic animals has also been tested. (Jenkins *et al.*, *Nature Biotechnol.* 14:975-81 (1996)).

[0005] All antibodies contain carbohydrate structures at conserved positions in the heavy chain constant regions, with each isotype possessing a distinct array of N-linked carbohydrate structures, which variably affect protein assembly, secretion or functional activity. (Wright, A., and Morrison, S. L., *Trends Biotech.* 15:26-32 (1997)). The structure of the attached N-linked carbohydrate

varies considerably, depending on the degree of processing, and can include high-mannose, multiply-branched as well as biantennary complex oligosaccharides. (Wright, A., and Morrison, S. L., *Trends Biotech.* 15:26-32 (1997)). Typically, there is heterogeneous processing of the core oligosaccharide structures attached at a particular glycosylation site such that even monoclonal antibodies exist as multiple glycoforms. Likewise, it has been shown that major differences in antibody glycosylation occur between cell lines, and even minor differences are seen for a given cell line grown under different culture conditions. (Lifely, M. R. *et al.*, *Glycobiology* 5(8):813-22 (1995)).

[0006] Unconjugated monoclonal antibodies (mAbs) can be useful medicines for the treatment of cancer, as demonstrated by the U.S. Food and Drug Administration's approval of Rituximab (Rituxan™; IDEC Pharmaceuticals, San Diego, CA, and Genentech Inc., San Francisco, CA), for the treatment of CD20 positive B-cell, low-grade or follicular Non-Hodgkin's lymphoma, and Trastuzumab (Herceptin™; Genentech Inc.) for the treatment of advanced breast cancer (Grillo-Lopez, A.-J., *et al.*, *Semin. Oncol.* 26:66-73 (1999); Goldenberg, M. M., *Clin. Ther.* 21:309-18 (1999)). The success of these products relies not only on their efficacy but also on their outstanding safety profiles (Grillo-Lopez, A.-J., *et al.*, *Semin. Oncol.* 26:66-73 (1999); Goldenberg, M. M., *Clin. Ther.* 21:309-18 (1999)). In spite of the achievements of these two drugs, there is currently a large interest in obtaining higher specific antibody activity than what is typically afforded by unconjugated mAb therapy.

[0007] One way to obtain large increases in potency, while maintaining a simple production process and potentially avoiding significant, undesirable side effects, is to enhance the natural, cell-mediated effector functions of mAbs by engineering their oligosaccharide component (Umaña, P. *et al.*, *Nature Biotechnol.* 17:176-180 (1999)). IgG1 type antibodies, the most commonly used antibodies in cancer immunotherapy, are glycoproteins that have a conserved N-linked glycosylation site at Asn297 in each CH2 domain. The two complex biantennary oligosaccharides attached to Asn297 are buried between the CH2

domains, forming extensive contacts with the polypeptide backbone, and their presence is essential for the antibody to mediate effector functions such as antibody dependent cellular cytotoxicity (ADCC) (Lifely, M. R., *et al.*, *Glycobiology* 5:813-822 (1995); Jefferis, R., *et al.*, *Immunol Rev.* 163:59-76 (1998); Wright, A. and Morrison, S. L., *Trends Biotechnol.* 15:26-32 (1997)).

[0008] The present inventors showed previously that overexpression in Chinese hamster ovary (CHO) cells of  $\beta(1,4)$ -N-acetylglucosaminyltransferase III (GnTIII), a glycosyltransferase catalyzing the formation of bisected oligosaccharides, significantly increases the *in vitro* ADCC activity of an anti-neuroblastoma chimeric monoclonal antibody (chCE7) produced by the engineered CHO cells. (See Umaña, P. *et al.*, *Nature Biotechnol.* 17:176-180 (1999), International Publication No. WO 99/54342, the entire contents of each of which are hereby incorporated by reference in their entirety). The antibody chCE7 belongs to a large class of unconjugated mAbs which have high tumor affinity and specificity, but have too little potency to be clinically useful when produced in standard industrial cell lines lacking the GnTIII enzyme (Umana, P., *et al.*, *Nature Biotechnol.* 17:176-180 (1999)). That study was the first to show that large increases of maximal *in vitro* ADCC activity could be obtained by increasing the proportion of constant region (Fc)-associated, bisected oligosaccharides above the levels found in naturally occurring antibodies. To determine if this finding could be extrapolated to an unconjugated mAb, which already has significant ADCC activity in the absence of bisected oligosaccharides, the present inventors have applied this technology to Rituximab, the anti-CD20, IDEC-C2B8 chimeric antibody. The present inventors have likewise applied the technology to the unconjugated anti-cancer mAb chG250.

## BRIEF SUMMARY OF THE INVENTION

[0009] The present inventors have now generated new glycosylation variants of the anti-CD20 monoclonal antibody (mAb) IDEC-C2B8 (Rituximab) and the anti-cancer mAb chG250 using genetically engineered mAb-producing cell lines that overexpress N-acetylglucosaminyltransferase III (GnTIII; EC 2.1.4.144) in a tetracycline regulated fashion. GnTIII is required for the synthesis of bisected oligosaccharides, which are found at low to intermediate levels in naturally-occurring human antibodies but are missing in mAbs produced in standard industrial cell lines. The new glycosylated versions outperformed Mabthera™ (the version of Rixtuximab marketed in Europe) and mouse-myeloma derived chG250 in biological (ADCC) activity. For example, a ten-fold lower amount of the variant carrying the highest levels of bisected oligosaccharides was required to reach the maximal ADCC activity as Mabthera™. For chG250, the variant carrying the highest levels of bisected oligosaccharides mediated significant ADCC activity at a 125-fold lower concentration than that required to detect even low ADCC activity by the unmodified control chG250. A clear correlation was found between the level of GnTIII expression and ADCC activity.

[0010] Accordingly, in one aspect the claimed invention is directed to a host cell engineered to produce a polypeptide having increased Fc-mediated cellular cytotoxicity by expression of at least one nucleic acid encoding  $\beta(1,4)$ -N-acetylglucosaminyltransferase III (GnT III), wherein the polypeptide produced by the host cell is selected from the group consisting of a whole antibody molecule, an antibody fragment, and a fusion protein which includes a region equivalent to the Fc region of an immunoglobulin, and wherein the GnT III is expressed in an amount sufficient to increase the proportion of said polypeptide carrying bisected hybrid oligosaccharides or galactosylated complex oligosaccharides or mixtures thereof in the Fc region relative to polypeptides carrying bisected complex oligosaccharides in the Fc region.

- [0011] In a preferred embodiment, the polypeptide is IgG or a fragment thereof, most preferably, IgG1 or a fragment thereof. In a further preferred embodiment, the polypeptide is a fusion protein that includes a region equivalent to the Fc region of a human IgG.
- [0012] In another aspect of the claimed invention, a nucleic acid molecule comprising at least one gene encoding GnTIII has been introduced into the host cell. In a preferred embodiment, at least one gene encoding GnTIII has been introduced into the host cell chromosome.
- [0013] Alternatively, the host cell has been engineered such that an endogenous GnT III gene is activated, for example, by insertion of a DNA element which increases gene expression into the host chromosome. In a preferred embodiment, the endogenous GnTIII has been activated by insertion of a promoter, an enhancer, a transcription factor binding site, a transposon, or a retroviral element or combinations thereof into the host cell chromosome. In another aspect, the host cell has been selected to carry a mutation triggering expression of an endogenous GnTIII. Preferably, the host cell is the CHO cell mutant lec 10.
- [0014] In a further preferred embodiment of the claimed invention, the at least one nucleic acid encoding a GnTIII is operably linked to a constitutive promoter element.
- [0015] In a further preferred embodiment, the host cell is a CHO cell, a BHK cell, a NS0 cell, a SP2/0 cell, or a hybridoma cell, a Y0 myeloma cell, a P3X63 mouse myeloma cell, a PER cell or a PER.C6 cell and said polypeptide is an anti-CD20 antibody. In another preferred embodiment, the host cell is a SP2/0 cell and the polypeptide is the monoclonal antibody chG250.
- [0016] In another aspect, the claimed invention is directed to a host cell that further comprises at least one transfected nucleic acid encoding an antibody molecule, an antibody fragment, or a fusion protein that includes a region equivalent to the Fc region of an immunoglobulin. In a preferred embodiment, the host cell comprises at least one transfected nucleic acid encoding an anti-CD20 antibody, the chimeric anti-human neuroblastoma monoclonal antibody



chCE7, the chimeric anti-human renal cell carcinoma monoclonal antibody chG250, the chimeric anti-human colon, lung, and breast carcinoma monoclonal antibody ING-1, the humanized anti-human 17-1A antigen monoclonal antibody 3622W94, the humanized anti-human colorectal tumor antibody A33, the anti-human melanoma antibody directed against GD3 ganglioside R24, or the chimeric anti-human squamous-cell carcinoma monoclonal antibody SF-25, an anti-human EGFR antibody, an anti-human EGFRvIII antibody, an anti-human PSMA antibody, and anti-human PSCA antibody, an anti-human CD22 antibody, an anti-human CD30 antibody, an anti-human CD33 antibody, an anti-human CD38 antibody, an anti-human CD40 antibody, an anti-human CD45 antibody, an anti-human CD52 antibody, an anti-human CD138 antibody, an anti-human HLA-DR variant antibody, an anti-human BpCAM antibody, an anti-human CEA antibody, an anti-human MUC1 antibody, an anti-human MUC1 core protein antibody, an anti-human aberrantly glycosylated MUC1 antibody, an antibody against human fibronectin variants containing the ED-B domain, and an anti-human HER2/neu antibody.

- [0017] In another aspect, the claimed invention is directed to a method for producing a polypeptide in a host cell comprising culturing any of the above-described the host cells under conditions which permit the production of said polypeptide having increased Fc-mediated cellular cytotoxicity. In a preferred embodiment, the method further comprises isolating said polypeptide having increased Fc-mediated cellular cytotoxicity.
- [0018] In a further preferred embodiment, the host cell comprises at least one nucleic acid encoding a fusion protein comprising a region equivalent to a glycosylated Fc region of an immunoglobulin.
- [0019] In a preferred embodiment, the proportion of bisected oligosaccharides in the Fc region of said polypeptides is greater than 50%, more preferably, greater than 70%. In another embodiment, the proportion of bisected hybrid oligosaccharides or galactosylated complex oligosaccharides or mixtures thereof

in the Fc region is greater than the proportion of bisected complex oligosaccharides in the Fc region of said polypeptide.

[0020] In a preferred aspect of the claimed method, the polypeptide is an anti-CD20 antibody and the anti-CD20 antibodies produced by said host cell have a glycosylation profile, as analyzed by MALDI/TOF-MS, that is substantially equivalent to that shown in FIG. 2E.

[0021] In another preferred aspect of the claimed method, the polypeptide is the chG250 monoclonal antibody and the chG250 antibodies produced by said host cell have a glycosylation profile, as analyzed by MALDI/TOF-MS, that is substantially equivalent to that shown in FIG. 7D.

[0022] In a further aspect, the claimed invention is directed to an antibody having increased antibody dependent cellular cytotoxicity (ADCC) produced by any of the methods described above. In preferred embodiments, the antibody is selected from the group consisting of an anti-CD20 antibody, chCE7, ch-G250, a humanized anti-HER2 monoclonal antibody, ING-1, 3622W94, SF-25, A33, and R24. Alternatively, the polypeptide can be an antibody fragment that includes a region equivalent to the Fc region of an immunoglobulin, having increased Fc-mediated cellular cytotoxicity produced by any of the methods described above.

[0023] In a further aspect, the claimed invention is directed to a fusion protein that includes a region equivalent to the Fc region of an immunoglobulin and having increased Fc-mediated cellular cytotoxicity produced by any of the methods described above.

[0024] In a further aspect, the claimed invention is directed to a pharmaceutical composition comprising the antibody, antibody fragment, or fusion protein of the invention and a pharmaceutically acceptable carrier.

[0025] In a further aspect, the claimed invention is directed to a method for the treatment of cancer comprising administering a therapeutically effective amount of said pharmaceutical composition to a patient in need thereof.

[0026] In a further aspect, the invention is directed to an improved method for treating an autoimmune disease produced in whole or in part by pathogenic autoantibodies based on B-cell depletion comprising administering a therapeutically effective amount of immunologically active antibody to a human subject in need thereof, the improvement comprising administering a therapeutically effective amount of an antibody having increased ADCC prepared as described above. In a preferred embodiment, the antibody is an anti-CD20 antibody. Examples of autoimmune diseases or disorders include, but are not limited to, immune-mediated thrombocytopenias, such as acute idiopathic thrombocytopenic purpura and chronic idiopathic thrombocytopenic purpura, dermatomyositis, Sydenham's chorea, lupus nephritis, rheumatic fever, polyglandular syndromes, Henoch-Schonlein purpura, post-streptococcal nephritis, erythema nodosum, Takayasu's arteritis, Addison's disease, erythema multiforme, polyarteritis nodosa, ankylosing spondylitis, Goodpasture's syndrome, thromboangitis obliterans, primary biliary cirrhosis, Hashimoto's thyroiditis, thyrotoxicosis, chronic active hepatitis, polymyositis/dermatomyositis, polychondritis, paronychia, Wegener's granulomatosis, membranous nephropathy, amyotrophic lateral sclerosis, tabes dorsalis, polymyalgia, pernicious anemia, rapidly progressive glomerulonephritis and fibrosing alveolitis, inflammatory responses such as inflammatory skin diseases including psoriasis and dermatitis (*e.g.* atopic dermatitis); systemic scleroderma and sclerosis; responses associated with inflammatory bowel disease (such as Crohn's disease and ulcerative colitis); respiratory distress syndrome (including adult respiratory distress syndrome; ARDS); dermatitis; meningitis; encephalitis; uveitis; colitis; glomerulonephritis; allergic conditions such as eczema and asthma and other conditions involving infiltration of T cells and chronic inflammatory responses; atherosclerosis; leukocyte adhesion deficiency; rheumatoid arthritis; systemic lupus erythematosus (SLE); diabetes mellitus (*e.g.* Type 1 diabetes mellitus or insulin dependent diabetes mellitus); multiple sclerosis; Reynaud's syndrome; autoimmune thyroiditis; allergic

encephalomyelitis; Sjorgen's syndrome; juvenile onset diabetes; and immune responses associated with acute and delayed hypersensitivity mediated by cytokines and T-lymphocytes typically found in tuberculosis, sarcoidosis, polymyositis, granulomatosis and vasculitis; pernicious anemia (Addison's disease); diseases involving leukocyte diapedesis; central nervous system (CNS) inflammatory disorder; multiple organ injury syndrome; hemolytic anemia (including, but not limited to cryoglobulinemia or Coombs positive anemia); myasthenia gravis; antigen-antibody complex mediated diseases; anti-glomerular basement membrane disease; antiphospholipid syndrome; allergic neuritis; Graves' disease; Lambert-Eaton myasthenic syndrome; pemphigoid bullous; pemphigus; autoimmune polyendocrinopathies; Reiter's disease; stiff-man syndrome; Behcet disease; giant cell arteritis; immune complex nephritis; IgA nephropathy; IgM polyneuropathies; immune thrombocytopenic purpura (ITP) or autoimmune thrombocytopenia etc. In this aspect of the invention, the antibodies of the invention are used to deplete the blood of normal B-cells for an extended period.

#### BRIEF DESCRIPTION OF THE FIGURES

[0027] FIG. 1. Indirect immunofluorescence assay showing the reactivity of the antibody preparation C2B8-25t to CD20 positive SB cells. Negative controls, including the HSB CD20 negative cell line and cells treated only with the secondary FITC-conjugated anti-human Fc polyclonal antibody are not shown.

[0028] FIG. 2A-2E. MALDI/TOF-MS spectra of the oligosaccharides derived from Mabthera™ (FIG. 2A), C2B8-nt (FIG. 2B), C2B8-2000t (FIG. 2C), C2B8-50t (FIG. 2D), and C2B8-25t (FIG. 2E) antibody samples. Oligosaccharides appear as  $[M+Na^+]$  and  $[M+K^+]$  ions. Oligosaccharide appearing in the first two spectra were derived from cell cultures that do not express GnTIII, whereas oligosaccharides in C, D, and E were derived from a single cell line expressing GnTIII at different levels (i.e. tetracycline concentrations).

- [0029] FIG. 3A and 3B. Illustration of a typical human IgG Fc-associated oligosaccharide structure (A) and partial N-linked glycosylation pathway (B). (FIG. 3A) The core of the oligosaccharide is composed of three mannose (M) and two N-acetylglucosamine (Gn) monosaccharide residues attached to Asn<sub>297</sub>. Galactose (G), fucose (F), and bisecting N-acetylglucosamine (Gn, boxed) can be present or absent. Terminal N-acetylneuraminic acid may be also present but it is not included in the figure. (FIG. 3B) Partial N-linked glycosylation pathway leading to the formation of the major oligosaccharide classes (dotted frames). Bisecting N-acetylglucosamine is denoted as Gn<sup>b</sup>. Subscript numbers indicate how many monosaccharide residues are present in each oligosaccharide. Each structure appears together with its sodium-associated [M+Na<sup>+</sup>] mass. The mass of those structures that contain fucose (f) are also included.
- [0030] FIG. 4A and 4B. ADCC activities of Rituximab glycosylation variants. The percentage of cytotoxicity was measured via lysis of <sup>51</sup>Cr labeled CD20-positive SB cells by human lymphocytes (E:T ratio of 100:1) mediated by different mAb concentrations. (FIG. 4A) Activity of C2B8 samples derived from a single cell line but produced at increasing GnTIII expression levels (i.e., decreasing tetracycline concentrations). The samples are C2B8-2000t, C2B8-50t, C2B8-25t, and C2B8-nt (control mAb derived from a clone that does not express GnTIII (FIG. 4B) ADCC activity of C2B8-50t and C2B8-25t compared to Mabthera™.
- [0031] FIG. 5. Western blot analysis of the seven GnTIII expressing clones and the wild type. 30 μg of each sample were loaded on a 8.75% SDS gel, transferred to a PVDF membrane and probed with the anti-c-myc monoclonal antibody (9E10). WT refers to wt-chG250-SP2/0 cells.
- [0032] FIG. 6. SDS polyacrylamide gel electrophoresis of resolved purified antibody samples.
- [0033] FIG. 7A-7D. MALDI/TOF-MS spectra of neutral oligosaccharide mixtures from chG250 mAb samples produced by clones expressing different

GnTIII levels and wt-chG250-SP2/0 cells: WT (FIG. 7A), 2F1 (FIG. 7B), 3D3 (FIG. 7C), 4E6 (FIG. 7D).

[0034] FIG. 8A-8D. MALDI/TOF-MS spectra of neutral oligosaccharide mixtures from chG250 mAb samples produced by clones expressing different GnTIII levels: 4E8, (FIG. 8A); 5G2, (FIG. 8B); 4G3, (FIG. 8C); 5H12, (FIG. 8D).

[0035] FIG. 9. In vitro ADCC assay of antibody samples derived from control wt-chG250-SP2/- cells and GnTIII transected clones 3D3 and 5H12.

#### DETAILED DESCRIPTION OF THE INVENTION

[0036] Terms are used herein as generally used in the art, unless otherwise defined as follows:

[0037] As used herein, the term *antibody* is intended to include whole antibody molecules, antibody fragments, or fusion proteins that include a region equivalent to the Fc region of an immunoglobulin.

[0038] As used herein, the term *region equivalent to the Fc region of an immunoglobulin* is intended to include naturally occurring allelic variants of the Fc region of an immunoglobulin as well as variants having alterations which produce substitutions, additions, or deletions but which do not decrease substantially the ability of the immunoglobulin to mediate antibody dependent cellular cytotoxicity. For example, one or more amino acids can be deleted from the N-terminus or C-terminus of the Fc region of an immunoglobulin without substantial loss of biological function. Such variants can be selected according to general rules known in the art so as to have minimal effect on activity. (See, e.g., Bowie, J. U. *et al.*, *Science* 247:1306-10 (1990).

[0039] As used herein, the term *glycoprotein-modifying glycosyl transferase* refers to  $\beta(1,4)$ -N-acetylglucosaminyltransferase III (GnTIII).

[0040] As used herein, the terms *engineer*, *engineered*, *engineering* and *glycosylation engineering* are considered to include any manipulation of the

glycosylation pattern of a naturally occurring polypeptide or fragment thereof. Glycosylation engineering includes metabolic engineering of the glycosylation machinery of a cell, including genetic manipulations of the oligosaccharide synthesis pathways to achieve altered glycosylation of glycoproteins expressed in cells. Furthermore, glycosylation engineering includes the effects of mutations and cell environment on glycosylation.

[0041] As used herein, the term *host cell* covers any kind of cellular system which can be engineered to generate modified glycoforms of proteins, protein fragments, or peptides of interest, including antibodies and antibody fragments. Typically, the host cells have been manipulated to express optimized levels of GnT III. Host cells include cultured cells, e.g., mammalian cultured cells, such as CHO cells, BHK cells, NS0 cells, SP2/0 cells, YO myeloma cells, P3X63 mouse myeloma cells, PER cells, PER.C6 cells or hybridoma cells, yeast cells, and insect cells, to name only a few, but also cells comprised within a transgenic animal or cultured tissue.

[0042] As used herein, the term *Fc-mediated cellular cytotoxicity* includes antibody-dependent cellular cytotoxicity and cellular cytotoxicity mediated by a soluble Fc-fusion protein containing a human Fc-region. It is an immune mechanism leading to the lysis of "antibody-targeted cells" by "human immune effector cells", wherein:

The "human immune effector cells" are a population of leukocytes that display Fc receptors on their surface through which they bind to the Fc-region of antibodies or of Fc-fusion proteins and perform effector functions. Such a population may include, but is not limited to, peripheral blood mononuclear cells (PBMC) and/or natural killer (NK) cells.

The "antibody-targeted cells" are cells bound by the antibodies or Fc-fusion proteins. The antibodies or Fc fusion-proteins bind to target cells via the protein part N-terminal to the Fc region.

[0043] As used herein, the term *increased Fc-mediated cellular cytotoxicity* is defined as either an increase in the number of "antibody-targeted cells" that are lysed in a given time, at a given concentration of antibody, or of Fc-fusion protein, in the medium surrounding the target cells, by the mechanism of Fc-mediated cellular cytotoxicity defined above, and/or a reduction in the concentration of antibody, or of Fc-fusion protein, in the medium surrounding the target cells, required to achieve the lysis of a given number of "antibody-targeted cells", in a given time, by the mechanism of Fc-mediated cellular cytotoxicity. The increase in Fc-mediated cellular cytotoxicity is relative to the cellular cytotoxicity mediated by the same antibody, or Fc-fusion protein, produced by the same type of host cells, using the same standard production, purification, formulation and storage methods, which are known to those skilled in the art, but that has not been produced by host cells engineered to express the glycosyltransferase GnTIII by the methods described herein.

[0044] By *antibody having increased antibody dependent cellular cytotoxicity (ADCC)* is meant an antibody having increased ADCC as determined by any suitable method known to those of ordinary skill in the art. One accepted *in vitro* ADCC assay is as follows:

- 1) the assay uses target cells that are known to express the target antigen recognized by the antigen-binding region of the antibody;
- 2) the assay uses human peripheral blood mononuclear cells (PBMCs), isolated from blood of a randomly chosen healthy donor, as effector cells;
- 3) the assay is carried out according to following protocol:
  - i) the PBMCs are isolated using standard density centrifugation procedures and are suspended at  $5 \times 10^6$  cells/ml in RPMI cell culture medium;
  - ii) the target cells are grown by standard tissue culture methods, harvested from the exponential growth phase with a viability higher than 90%, washed in RPMI cell culture medium, labelled with 100 micro-Curies



of  $^{51}\text{Cr}$ , washed twice with cell culture medium, and resuspended in cell culture medium at a density of  $10^5$  cells/ml;

iii) 100 microliters of the final target cell suspension above are transferred to each well of a 96-well microtiter plate;

iv) the antibody is serially-diluted from 4000 ng/ml to 0.04 ng/ml in cell culture medium and 50 microliters of the resulting antibody solutions are added to the target cells in the 96-well microtiter plate, testing in triplicate various antibody concentrations covering the whole concentration range above;

v) for the maximum release (MR) controls, 3 additional wells in the plate containing the labelled target cells, receive 50 microliters of a 2% (V/V) aqueous solution of non-ionic detergent (Nonidet, Sigma, St. Louis), instead of the antibody solution (point iv above);

vi) for the spontaneous release (SR) controls, 3 additional wells in the plate containing the labelled target cells, receive 50 microliters of RPMI cell culture medium instead of the antibody solution (point iv above);

vii) the 96-well microtiter plate is then centrifuged at  $50 \times g$  for 1 minute and incubated for 1 hour at  $4^\circ\text{C}$ ;

viii) 50 microliters of the PBMC suspension (point i above) are added to each well to yield an effector:target cell ratio of 25:1 and the plates are placed in an incubator under 5%  $\text{CO}_2$  atmosphere at  $37^\circ\text{C}$  for 4 hours;

ix) the cell-free supernatant from each well is harvested and the experimentally released radioactivity (ER) is quantified using a gamma counter;

x) the percentage of specific lysis is calculated for each antibody concentration according to the formula  $(\text{ER}-\text{MR})/(\text{MR}-\text{SR}) \times 100$ , where ER is the average radioactivity quantified (see point ix above) for that antibody concentration, MR is the average radioactivity quantified (see point ix above) for the MR controls (see point v above), and SR is the average

radioactivity quantified (see point ix above) for the SR controls (see point vi above);

4) "increased ADCC" is defined as either an increase in the maximum percentage of specific lysis observed within the antibody concentration range tested above, and/or a reduction in the concentration of antibody required to achieve one half of the maximum percentage of specific lysis observed within the antibody concentration range tested above. The increase in ADCC is relative to the ADCC, measured with the above assay, mediated by the same antibody, produced by the same type of host cells, using the same standard production, purification, formulation and storage methods, which are known to those skilled in the art, but that has not been produced by host cells engineered to overexpress the glycosyltransferase GnTIII.

[0045] As used herein, the term *anti-CD20 antibody* is intended to mean an antibody which specifically recognizes a cell surface non-glycosylated phosphoprotein of 35,000 Daltons, typically designated as the human B lymphocyte restricted differentiation antigen Bp35, commonly referred to as CD20.

#### Identification and Generation of Nucleic Acids Encoding A Protein for Which Modification of The Glycosylation Pattern Is Desired

[0046] The present invention provides methods for the generation and use of host cell systems for the production of glycoforms of antibodies or antibody fragments or fusion proteins which include antibody fragments with increased antibody-dependent cellular cytotoxicity. Identification of target epitopes and generation of antibodies having potential therapeutic value, for which modification of the glycosylation pattern is desired, and isolation of their respective coding nucleic acid sequence is within the scope of the invention.

[0047] Various procedures known in the art may be used for the production of antibodies to target epitopes of interest. Such antibodies include but are not

limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments and fragments produced by an Fab expression library. Such antibodies may be useful, *e.g.*, as diagnostic or therapeutic agents. As therapeutic agents, neutralizing antibodies, *i.e.*, those which compete for binding with a ligand, substrate or adapter molecule, are of especially preferred interest.

[0048] For the production of antibodies, various host animals are immunized by injection with the target protein of interest including, but not limited to, rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, saponin, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (*bacille Calmette-Guerin*) and *Corynebacterium parvum*.

[0049] Monoclonal antibodies to the target of interest may be prepared using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique originally described by Kohler and Milstein, *Nature* 256:495-97 (1975), the human B-cell hybridoma technique (Kosbor *et al.*, *Immunology Today* 4:72 (1983); Cote *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 80:2026-30 (1983) and the EBV-hybridoma technique (Cole *et al.*, *Monoclonal Antibodies and Cancer Therapy* 77-96 (Alan R. Liss, Inc., 1985)). In addition, techniques developed for the production of "chimeric antibodies" (Morrison *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 81:6851-55 (1984); Neuberger *et al.*, *Nature* 312:604-08 (1984) ; Takeda *et al.*, *Nature* 314:452-54 (1985) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. Alternatively, techniques described for the production of single chain antibodies (U.S. Patent No. 4,946,778) can be adapted to produce single chain antibodies having a desired specificity.

[0050] Antibody fragments which contain specific binding sites of the target protein of interest may be generated by known techniques. For example, such fragments include, but are not limited to,  $F(ab')_2$  fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the  $F(ab')_2$  fragments. Alternatively, Fab expression libraries may be constructed (Huse *et al.*, *Science* 246:1275-81 (1989) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity to the target protein of interest.

[0051] Once an antibody or antibody fragment has been identified for which modification in the glycosylation pattern are desired, the coding nucleic acid sequence is identified and isolated using techniques well known in the art.

a. Generation Of Cell Lines For The Production Of Proteins With Altered Glycosylation Pattern

[0052] The present invention provides host cell expression systems for the generation of proteins having modified glycosylation patterns. In particular, the present invention provides host cell systems for the generation of glycoforms of proteins having an improved therapeutic value. Therefore, the invention provides host cell expression systems selected or engineered to increase the expression of a glycoprotein-modifying glycosyltransferase, namely  $\beta(1,4)$ -N-acetylglucosaminyltransferase III (GnTIII). Specifically, such host cell expression systems may be engineered to comprise a recombinant nucleic acid molecule encoding GnTIII, operatively linked to a constitutive or regulated promoter system. Alternatively, host cell expression systems may be employed that naturally produce, are induced to produce, and/or are selected to produce GnTIII.

[0053] In one specific embodiment, the present invention provides a host cell that has been engineered to express at least one nucleic acid encoding GnTIII.

In one aspect, the host cell is transformed or transfected with a nucleic acid molecule comprising at least one gene encoding GnTIII. In an alternate aspect, the host cell has been engineered and/or selected in such way that endogenous GnTIII is activated. For example, the host cell may be selected to carry a mutation triggering expression of endogenous GnTIII. In one specific embodiment, the host cell is a CHO lec10 mutant. Alternatively, the host cell may be engineered such that endogenous GnTIII is activated. In again another alternative, the host cell is engineered such that endogenous GnTIII has been activated by insertion of a constitutive promoter element, a transposon, or a retroviral element into the host cell chromosome.

[0054] Generally, any type of cultured cell line can be used as a background to engineer the host cell lines of the present invention. In a preferred embodiment, CHO cells, BHK cells, NS0 cells, SP2/0 cells, YO myeloma cells, P3X63 mouse myeloma cells, PER cells, PER.C6 cells or hybridoma cells, yeast cells, or insect cells are used as the background cell line to generate the engineered host cells of the invention.

[0055] The invention is contemplated to encompass any engineered host cells expressing GnTIII as defined herein:

[0056] One or several nucleic acids encoding GnTIII may be expressed under the control of a constitutive promoter or, alternately, a regulated expression system. Suitable regulated expression systems include, but are not limited to, a tetracycline-regulated expression system, an ecdysone-inducible expression system, a lac-switch expression system, a glucocorticoid-inducible expression system, a temperature-inducible promoter system, and a metallothionein metal-inducible expression system. If several different nucleic acids encoding GnTIII are comprised within the host cell system, some of them may be expressed under the control of a constitutive promoter, while others are expressed under the control of a regulated promoter. The maximal expression level is considered to be the highest possible level of stable GnTIII expression that does not have a significant adverse effect on cell growth rate, and will be determined using

routine experimentation. Expression levels are determined by methods generally known in the art, including Western blot analysis using a GnTIII specific antibody, Northern blot analysis using a GnTIII specific nucleic acid probe, or measurement of enzymatic activity. Alternatively, a lectin may be employed which binds to biosynthetic products of the GnTIII, for example, E<sub>4</sub>-PHA lectin. In a further alternative, the nucleic acid may be operatively linked to a reporter gene; the expression levels of the GnTIII are determined by measuring a signal correlated with the expression level of the reporter gene. The reporter gene may transcribed together with the nucleic acid(s) encoding said GnTIII as a single mRNA molecule; their respective coding sequences may be linked either by an internal ribosome entry site (IRES) or by a cap-independent translation enhancer (CITE). The reporter gene may be translated together with at least one nucleic acid encoding said GnTIII such that a single polypeptide chain is formed. The nucleic acid encoding the GnTIII may be operatively linked to the reporter gene under the control of a single promoter, such that the nucleic acid encoding the GnTIII and the reporter gene are transcribed into an RNA molecule which is alternatively spliced into two separate messenger RNA (mRNA) molecules; one of the resulting mRNAs is translated into said reporter protein, and the other is translated into said GnTIII.

[0057] If several different nucleic acids encoding GnTIII are expressed, they may be arranged in such way that they are transcribed as one or as several mRNA molecules. If they are transcribed as a single mRNA molecule, their respective coding sequences may be linked either by an internal ribosome entry site (IRES) or by a cap-independent translation enhancer (CITE). They may be transcribed from a single promoter into an RNA molecule which is alternatively spliced into several separate messenger RNA (mRNA) molecules, which then are each translated into their respective encoded GnTIII.

[0058] In other embodiments, the present invention provides host cell expression systems for the generation of therapeutic antibodies, having an increased antibody-dependent cellular cytotoxicity, and cells which display the IgG Fc

region on the surface to promote Fc-mediated cytotoxicity. Generally, the host cell expression systems have been engineered and/or selected to express nucleic acids encoding the antibody for which the production of altered glycoforms is desired, along with at least one nucleic acid encoding GnTIII. In one embodiment, the host cell system is transfected with at least one gene encoding GnTIII. Typically, the transfected cells are selected to identify and isolate clones that stably express the GnTIII. In another embodiment, the host cell has been selected for expression of endogenous GnTIII. For example, cells may be selected carrying mutations which trigger expression of otherwise silent GnTIII. For example, CHO cells are known to carry a silent GnT III gene that is active in certain mutants, *e.g.*, in the mutant Lec10. Furthermore, methods known in the art may be used to activate silent GnTIII, including the insertion of a regulated or constitutive promoter, the use of transposons, retroviral elements, etc. Also the use of gene knockout technologies or the use of ribozyme methods may be used to tailor the host cell's GnTIII expression level, and is therefore within the scope of the invention.

[0059] Any type of cultured cell line can be used as background to engineer the host cell lines of the present invention. In a preferred embodiment, CHO cells, BHK cells, NS0 cells, SP2/0 cells, YO myeloma cells, P3X63 mouse myeloma cells, PER cells, PER.C6 cells or hybridoma cells, yeast cell, or insect cells may be used. Typically, such cell lines are engineered to further comprise at least one transfected nucleic acid encoding a whole antibody molecule, an antibody fragment, or a fusion protein that includes a region equivalent to the Fc region of an immunoglobulin. In an alternative embodiment, a hybridoma cell line expressing a particular antibody of interest is used as background cell line to generate the engineered host cells of the invention.

[0060] Typically, at least one nucleic acid in the host cell system encodes GnT III.

[0061] One or several nucleic acids encoding GnTIII may be expressed under the control of a constitutive promoter, or alternately, a regulated expression system.

Suitable regulated expression systems include, but are not limited to, a tetracycline-regulated expression system, an ecdysone-inducible expression system, a lac-switch expression system, a glucocorticoid-inducible expression system, a temperature-inducible promoter system, and a metallothionein metal-inducible expression system. If several different nucleic acids encoding GnTIII are comprised within the host cell system, some of them may be expressed under the control of a constitutive promoter, while others are expressed under the control of a regulated promoter. The maximal expression level is considered to be the highest possible level of stable GnTIII expression that does not have a significant adverse effect on cell growth rate, and will be determined using routine experimentation. Expression levels are determined by methods generally known in the art, including Western blot analysis using a GnTIII specific antibody, Northern blot analysis using a GnTIII specific nucleic acid probe, or measurement of GnTIII enzymatic activity. Alternatively, a lectin may be employed which binds to biosynthetic products of GnTIII, for example, E<sub>4</sub>-PHA lectin. In a further alternative, the nucleic acid may be operatively linked to a reporter gene; the expression levels of the glycoprotein-modifying glycosyl transferase are determined by measuring a signal correlated with the expression level of the reporter gene. The reporter gene may transcribed together with the nucleic acid(s) encoding said glycoprotein-modifying glycosyl transferase as a single mRNA molecule; their respective coding sequences may be linked either by an internal ribosome entry site (IRES) or by a cap-independent translation enhancer (CITE). The reporter gene may be translated together with at least one nucleic acid encoding GnTIII such that a single polypeptide chain is formed. The nucleic acid encoding the GnTIII may be operatively linked to the reporter gene under the control of a single promoter, such that the nucleic acid encoding the GnTIII and the reporter gene are transcribed into an RNA molecule which is alternatively spliced into two separate messenger RNA (mRNA) molecules; one of the resulting mRNAs is translated into said reporter protein, and the other is translated into said GnTIII.



[0062] If several different nucleic acids encoding a GnTIII are expressed, they may be arranged in such way that they are transcribed as one or as several mRNA molecules. If they are transcribed as single mRNA molecule, their respective coding sequences may be linked either by an internal ribosome entry site (IRES) or by a cap-independent translation enhancer (CITE). They may be transcribed from a single promoter into an RNA molecule which is alternatively spliced into several separate messenger RNA (mRNA) molecules, which then are each translated into their respective encoded GnTIII.

i. Expression Systems

[0063] Methods which are well known to those skilled in the art can be used to construct expression vectors containing the coding sequence of the protein of interest and the coding sequence of the GnTIII and appropriate transcriptional/translational control signals. These methods include *in vitro* recombinant DNA techniques, synthetic techniques and *in vivo* recombination/genetic recombination. See, for example, the techniques described in Maniatis *et al.*, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. (1989) and Ausubel *et al.*, Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, N.Y (1989).

[0064] A variety of host-expression vector systems may be utilized to express the coding sequence of the protein of interest and the coding sequence of the GnTIII. Preferably, mammalian cells are used as host cell systems transfected with recombinant plasmid DNA or cosmid DNA expression vectors containing the coding sequence of the protein of interest and the coding sequence of the GnTIII. Most preferably, CHO cells, BHK cells, NS0 cells, SP2/0 cells, YO myeloma cells, P3X63 mouse myeloma cells, PER cells, PER.C6 cells or hybridoma cells, yeast cells, or insect cells are used as host cell system. In alternate embodiments, other eukaryotic host cell systems may be contemplated, including, yeast cells transformed with recombinant yeast expression vectors containing the coding

sequence of the protein of interest and the coding sequence of the GnTIII; insect cell systems infected with recombinant virus expression vectors (*e.g.*, baculovirus) containing the coding sequence of the protein of interest and the coding sequence of the GnTIII; plant cell systems infected with recombinant virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing the coding sequence of the protein of interest and the coding sequence of the GnTIII; or animal cell systems infected with recombinant virus expression vectors (*e.g.*, adenovirus, vaccinia virus) including cell lines engineered to contain multiple copies of the DNA encoding the protein of interest and the coding sequence of the GnTIII either stably amplified (CHO/dhfr) or unstably amplified in double-minute chromosomes (*e.g.*, murine cell lines).

[0065] For the methods of this invention, stable expression is generally preferred to transient expression because it typically achieves more reproducible results and also is more amenable to large scale production. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with the respective coding nucleic acids controlled by appropriate expression control elements (*e.g.*, promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.); and a selectable marker. Following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows selection of cells which have stably integrated the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines.

[0066] A number of selection systems may be used, including, but not limited to, the herpes simplex virus thymidine kinase (Wigler *et al.*, *Cell* 11:223 (1977)), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, *Proc. Natl. Acad. Sci. USA* 48:2026 (1962)), and adenine phosphoribosyltransferase (Lowy *et al.*, *Cell* 22:817 (1980)) genes, which can be employed in tk<sup>-</sup>, hgp<sup>+</sup> or

aprt cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler *et al.*, *Natl. Acad. Sci. USA* 77:3567 (1989); O'Hare *et al.*, *Proc. Natl. Acad. Sci. USA* 78:1527 (1981)); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, *Proc. Natl. Acad. Sci. USA* 78:2072 (1981)); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin *et al.*, *J. Mol. Biol.* 150:1 (1981)); and hygro, which confers resistance to hygromycin (Santerre *et al.*, *Gene* 30:147 (1984) genes. Recently, additional selectable genes have been described, namely trpB, which allows cells to utilize indole in place of tryptophan; hisD, which allows cells to utilize histinol in place of histidine (Hartman & Mulligan, *Proc. Natl. Acad. Sci. USA* 85:8047 (1988)); the glutamine synthase system; and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue, in: *Current Communications in Molecular Biology*, Cold Spring Harbor Laboratory ed. (1987)).

ii. Identification Of Transfectants Or Transformants That Express The Protein Having A Modified Glycosylation Pattern

[0067] The host cells which contain the coding sequence and which express the biologically active gene products may be identified by at least four general approaches; (a) DNA-DNA or DNA-RNA hybridization; (b) the presence or absence of "marker" gene functions; (c) assessing the level of transcription as measured by the expression of the respective mRNA transcripts in the host cell; and (d) detection of the gene product as measured by immunoassay or by its biological activity.

[0068] In the first approach, the presence of the coding sequence of the protein of interest and the coding sequence of the GnTIII inserted in the expression vector can be detected by DNA-DNA or DNA-RNA hybridization using probes

comprising nucleotide sequences that are homologous to the respective coding sequences, respectively, or portions or derivatives thereof.

[0069] In the second approach, the recombinant expression vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (*e.g.*, thymidine kinase activity, resistance to antibiotics, resistance to methotrexate, transformation phenotype, occlusion body formation in baculovirus, etc.). For example, if the coding sequence of the protein of interest and the coding sequence of the GnTIII are inserted within a marker gene sequence of the vector, recombinants containing the respective coding sequences can be identified by the absence of the marker gene function. Alternatively, a marker gene can be placed in tandem with the coding sequences under the control of the same or different promoter used to control the expression of the coding sequences. Expression of the marker in response to induction or selection indicates expression of the coding sequence of the protein of interest and the coding sequence of the GnTIII.

[0070] In the third approach, transcriptional activity for the coding region of the protein of interest and the coding sequence of the GnTIII can be assessed by hybridization assays. For example, RNA can be isolated and analyzed by Northern blot using a probe homologous to the coding sequences of the protein of interest and the coding sequence of the GnTIII or particular portions thereof. Alternatively, total nucleic acids of the host cell may be extracted and assayed for hybridization to such probes.

[0071] In the fourth approach, the expression of the protein products of the protein of interest and the coding sequence of the GnTIII can be assessed immunologically, for example by Western blots, immunoassays such as radioimmuno-precipitation, enzyme-linked immunoassays and the like. The ultimate test of the success of the expression system, however, involves the detection of the biologically active gene products.

- b. Generation And Use Of Proteins And Protein Fragments Having Altered Glycosylation Patterns
  - i. Generation And Use Of Antibodies Having Increased Antibody-Dependent Cellular Cytotoxicity

[0072] In preferred embodiments, the present invention provides glycoforms of antibodies and antibody fragments having increased antibody-dependent cellular cytotoxicity.

[0073] Clinical trials of unconjugated monoclonal antibodies (mAbs) for the treatment of some types of cancer have recently yielded encouraging results. Dillman, *Cancer Biother. & Radiopharm.* 12:223-25 (1997); Deo *et al.*, *Immunology Today* 18:127 (1997). A chimeric, unconjugated IgG1 has been approved for low-grade or follicular B-cell non-Hodgkin's lymphoma Dillman, *Cancer Biother. & Radiopharm.* 12:223-25 (1997), while another unconjugated mAb, a humanized IgG1 targeting solid breast tumors, has also been showing promising results in phase III clinical trials. Deo *et al.*, *Immunology Today* 18:127 (1997). The antigens of these two mAbs are highly expressed in their respective tumor cells and the antibodies mediate potent tumor destruction by effector cells *in vitro* and *in vivo*. In contrast, many other unconjugated mAbs with fine tumor specificities cannot trigger effector functions of sufficient potency to be clinically useful. Frost *et al.*, *Cancer* 80:317-33 (1997); Surfus *et al.*, *J. Immunother.* 19:184-91 (1996). For some of these weaker mAbs, adjunct cytokine therapy is currently being tested. Addition of cytokines can stimulate antibody-dependent cellular cytotoxicity (ADCC) by increasing the activity and number of circulating lymphocytes. Frost *et al.*, *Cancer* 80:317-33 (1997); Surfus *et al.*, *J. Immunother.* 19:184-91 (1996). ADCC, a lytic attack on antibody-targeted cells, is triggered upon binding of leukocyte receptors to the constant region (Fc) of antibodies. Deo *et al.*, *Immunology Today* 18:127 (1997).

[0074] A different, but complementary, approach to increase ADCC activity of unconjugated IgG1s is to engineer the Fc region of the antibody to increase its

affinity for the lymphocyte receptors (FcγRs). Protein engineering studies have shown that FcγRs interact with the lower hinge region of the IgG CH2 domain. Lund *et al.*, *J. Immunol.* 157:4963-69 (1996). However, FcγR binding also requires the presence of oligosaccharides covalently attached at the conserved Asn 297 in the CH2 region. Lund *et al.*, *J. Immunol.* 157:4963-69 (1996); Wright and Morrison, *Trends Biotech.* 15:26-31 (1997), suggesting that either oligosaccharide and polypeptide both directly contribute to the interaction site or that the oligosaccharide is required to maintain an active CH2 polypeptide conformation. Modification of the oligosaccharide structure can therefore be explored as a means to increase the affinity of the interaction.

[0075] An IgG molecule carries two N-linked oligosaccharides in its Fc region, one on each heavy chain. As any glycoprotein, an antibody is produced as a population of glycoforms which share the same polypeptide backbone but have different oligosaccharides attached to the glycosylation sites. The oligosaccharides normally found in the Fc region of serum IgG are of complex bi-antennary type (Wormald *et al.*, *Biochemistry* 36:130-38 (1997), with low level of terminal sialic acid and bisecting N-acetylglucosamine (GlcNAc), and a variable degree of terminal galactosylation and core fucosylation. Some studies suggest that the minimal carbohydrate structure required for FcγR binding lies within the oligosaccharide core. Lund *et al.*, *J. Immunol.* 157:4963-69 (1996) The removal of terminal galactoses results in approximately a two-fold reduction in ADCC activity, indicating a role for these residues in FcγR receptor binding. Lund *et al.*, *J. Immunol.* 157:4963-69 (1996)

[0076] The mouse- or hamster-derived cell lines used in industry and academia for production of unconjugated therapeutic mAbs normally attach the required oligosaccharide determinants to Fc sites. IgGs expressed in these cell lines lack, however, the bisecting GlcNAc found in low amounts in serum IgGs. Lifely *et al.*, *Glycobiology* 318:813-22 (1995). In contrast, it was recently observed that a rat myeloma-produced, humanized IgG1 (CAMPATH-1H) carried a bisecting GlcNAc in some of its glycoforms. Lifely *et al.*, *Glycobiology* 318:813-22

(1995). The rat cell-derived antibody reached a similar *in vitro* ADCC activity as CAMPATH-1H antibodies produced in standard cell lines, but at significantly lower antibody concentrations.

[0077] The CAMPATH antigen is normally present at high levels on lymphoma cells, and this chimeric mAb has high ADCC activity in the absence of a bisecting GlcNAc. Lifely *et al.*, *Glycobiology* 318:813-22 (1995). In the N-linked glycosylation pathway, a bisecting GlcNAc is added by the enzyme  $\beta(1,4)$ -N-acetylglucosaminyltransferase III (GnT III). Schachter, *Biochem. Cell Biol.* 64:163-81 (1986).

[0078] The present inventors used a single antibody-producing CHO cell line, that was previously engineered to express, in an externally-regulated fashion, different levels of a cloned GnT III gene. This approach established for the first time a rigorous correlation between expression of GnT III and the ADCC activity of the modified antibody.

[0079] The present inventors previously showed that C2B8 antibody modified according to the disclosed method had an about sixteen-fold higher ADCC activity than the standard, unmodified C2B8 antibody produced under identical cell culture and purification conditions. Briefly, a C2B8 antibody sample expressed in CHO-tTA-C2B8 cells that do not have GnT III expression showed a cytotoxic activity of about 31% (at 1  $\mu$ g/ml antibody concentration), measured as *in vitro* lysis of SB cells (CD20+) by human lymphocytes. In contrast, C2B8 antibody derived from a CHO cell culture expressing GnT III at a basal, largely repressed level showed at 1  $\mu$ g/ml antibody concentration a 33% increase in ADCC activity against the control at the same antibody concentration. Moreover, increasing the expression of GnT III produced a large increase of almost 80% in the maximal ADCC activity (at 1  $\mu$ g/ml antibody concentration) compared to the control at the same antibody concentration. (See International Publication No. WO 99/54342, the entire contents of which are hereby incorporated by reference.)

[0080] Further antibodies of the invention having increased antibody-dependent cellular cytotoxicity include, but are not limited to, anti-human neuroblastoma monoclonal antibody (chCE7) produced by the methods of the invention, a chimeric anti-human renal cell carcinoma monoclonal antibody (ch-G250) produced by the methods of the invention, a humanized anti-HER2 monoclonal antibody (*e.g.*, Trastuzumab (HERCEPTIN)) produced by the methods of the invention, a chimeric anti-human colon, lung, and breast carcinoma monoclonal antibody (ING-1) produced by the methods of the invention, a humanized anti-human 17-1A antigen monoclonal antibody (3622W94) produced by the methods of the invention, a humanized anti-human colorectal tumor antibody (A33) produced by the methods of the invention, an anti-human melanoma antibody (R24) directed against GD3 ganglioside produced by the methods of the invention, and a chimeric anti-human squamous-cell carcinoma monoclonal antibody (SF-25) produced by the methods of the invention, an anti-human small cell lung carcinoma monoclonal antibody (BEC2, ImClone Systems, Merck KgaA) produced by the methods of the invention, an anti-human non-Hodgkin's lymphoma monoclonal antibody (Bexxar (tositumomab, Coulter Pharmaceuticals), Oncolym (Techniclone, Alpha Therapeutic)) produced by the methods of the invention, an anti-human squamous cell head and neck carcinoma monoclonal antibody (C225, ImClone Systems) prepared by the methods of the invention, an anti-human rectal and colon carcinoma monoclonal antibody (Panorex (edrecolomab), Centocor, Glaxo Wellcome) prepared by the methods of the invention, an anti-human ovarian carcinoma monoclonal antibody (Theragyn, Antisoma) produced by the methods of the invention, an anti-human acute myelogenous leukemia carcinoma monoclonal antibody (SmartM195, Protein Design Labs, Kanebo) produced by the methods of the invention, an anti-human malignant glioma monoclonal antibody (Cotara, Techniclone, Cambridge Antibody Technology) produced by the methods of the invention, an anti-human B cell non-Hodgkins lymphoma monoclonal antibody (IDEC-Y2B8, IDEC Pharmaceuticals) produced by the methods of the invention, an anti-human solid



tumors monoclonal antibody (CEA-Cide, Immunomedics) produced by the methods of the invention, an anti-human colorectal carcinoma monoclonal antibody (Iodine 131-MN-14, Immunomedics) produced by the methods of the invention, an anti-human ovary, kidney, breast, and prostate carcinoma monoclonal antibody (MDX-210, Medarex, Novartis) produced by the methods of the invention, an anti-human colorectal and pancreas carcinoma monoclonal antibody (TTMA, Pharmacia & Upjohn) produced by the methods of the invention, an anti-human TAG-72 expressing carcinoma monoclonal antibody (MDX-220, Medarex) produced by the methods of the invention, an anti-human EGFr-expressing carcinoma monoclonal antibody (MDX-447) produced by the methods of the invention, Anti-VEGF monoclonal antibody (Genentech) produced by the methods of the invention, an anti-human breast, lung, prostate and pancreas carcinoma and malignant melanoma monoclonal antibody (BrevaRex, AltaRex) produced by the methods of the invention, and an anti-human acute myelogenous leukemia monoclonal antibody (Monoclonal Antibody Conjugate, Immunex) produced by the methods of the invention. In addition, the invention is directed to antibody fragment and fusion proteins comprising a region that is equivalent to the Fc region of immunoglobulins.

ii. Generation And Use Of Fusion Proteins Comprising A Region Equivalent To An Fc Region Of An Immunoglobulin That Promote Fc-Mediated Cytotoxicity

[0081] As discussed above, the present invention relates to a method for increasing the ADCC activity of therapeutic antibodies. This is achieved by engineering the glycosylation pattern of the Fc region of such antibodies, in particular by maximizing the proportion of antibody molecules carrying bisected complex oligosaccharides and bisected hybrid oligosaccharides N-linked to the conserved glycosylation sites in their Fc regions. This strategy can be applied to increase Fc-mediated cellular cytotoxicity against undesirable cells mediated by any molecule carrying a region that is an equivalent to the Fc region of an

immunoglobulin, not only by therapeutic antibodies, since the changes introduced by the engineering of glycosylation affect only the Fc region and therefore its interactions with the Fc receptors on the surface of effector cells involved in the ADCC mechanism. Fc-containing molecules to which the presently disclosed methods can be applied include, but are not limited to, (a) soluble fusion proteins made of a targeting protein domain fused to the N-terminus of an Fc-region (Chamov and Ashkenazi, *Trends Biotech.* 14: 52 (1996)) and (b) plasma membrane-anchored fusion proteins made of a type II transmembrane domain that localizes to the plasma membrane fused to the N-terminus of an Fc region (Stabila, P.F., *Nature Biotech.* 16: 1357 (1998)).

[0082] In the case of soluble fusion proteins (a) the targeting domain directs binding of the fusion protein to undesirable cells such as cancer cells, *i.e.*, in an analogous fashion to therapeutic antibodies. The application of presently disclosed method to enhance the Fc-mediated cellular cytotoxic activity mediated by these molecules would therefore be identical to the method applied to therapeutic antibodies.

[0083] In the case of membrane-anchored fusion proteins (b) the undesirable cells in the body have to express the gene encoding the fusion protein. This can be achieved either by gene therapy approaches, *i.e.*, by transfecting the cells *in vivo* with a plasmid or viral vector that directs expression of the fusion protein-encoding gene to undesirable cells, or by implantation in the body of cells genetically engineered to express the fusion protein on their surface. The later cells would normally be implanted in the body inside a polymer capsule (encapsulated cell therapy) where they cannot be destroyed by an Fc-mediated cellular cytotoxicity mechanism. However should the capsule device fail and the escaping cells become undesirable, then they can be eliminated by Fc-mediated cellular cytotoxicity. Stabila *et al.*, *Nature Biotech.* 16: 1357 (1998). In this case, the presently disclosed method would be applied either by incorporating into the gene therapy vector an additional gene expression cassette directing adequate or maximal expression levels of GnT III or by engineering the cells to

be implanted to express adequate or maximal levels of GnT III. In both cases, the aim of the disclosed method is to increase or maximize the proportion of surface-displayed Fc regions carrying bisected complex oligosaccharides and/or bisected hybrid oligosaccharides.

[0084] The examples below explain the invention in more detail. The following preparations and examples are given to enable those skilled in the art to more clearly understand and to practice the present invention. The present invention, however, is not limited in scope by the exemplified embodiments, which are intended as illustrations of single aspects of the invention only, and methods which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

#### EXAMPLE 1

New Versions of the Chimeric Anti-CD20 Antibody IDEC-C2B8  
Having Enhanced Antibody-Dependent Cellular Cytotoxicity  
Obtained by Glycosylation Engineering of an IDEC-CEB8  
Producing Cell Line

[0085] Synthesis of *VH* and *VL* coding regions of IDEC-C2B8 and construction of mammalian expression vectors. cDNAs encoding the *VH* and *VL* regions of IDEC-C2B8 antibody were assembled from a set of overlapping single-stranded oligonucleotides in a one-step process using PCR (Kobayashi, N., *et al.*, *Biotechniques* 23:500-503 (1997)). The original sequence data coding for IDEC-C2B8 *VL* and *VH* were obtained from a published international patent application (International Publication Number: WO 94/11026). Assembled *VL* and *VH* cDNA fragments were subcloned into pBluescriptIIKS(+), sequenced and directly joined by ligation to the human constant light (Ig $\kappa$ ) and heavy (IgG1) chain cDNAs, respectively, using unique restriction sites introduced at the

variable and constant region junctions without altering the original amino acid residue sequence (Umana, P., *et al.*, *Nat Biotechnol.* 17:176-180 (1999); Reff, M. E., *et al.*, *Blood* 83:435-445 (1994)). Each full-length cDNA was separately subcloned into pcDNA3.1(+) (Invitrogen, Leek, The Netherlands) yielding mammalian expression vectors for chimeric C2B8 light (pC2B8L) and heavy (pC2B8H) chains.

[0086] *Production of IDEC-C2B8 in CHO cells expressing different levels of GnTIII.* Establishment of two CHO cell lines, CHO-tet-GnTIII expressing different levels of GnTIII depending on the tetracycline concentration in the culture medium; and CHO-tTA, the parental cell line that does not express GnTIII has been described previously (Umana, P., *et al.*, *Nat Biotechnol.* 17:176-180 (1999); Umana, P., *et al.*, *Biotechnol Bioeng.* 65:542-549 (1999)). Each cell line was cotransfected with vectors pC2B8L, pC2B8H, and pZeoSV2(+) (for Zeocin resistance; Invitrogen, Leek, The Netherlands) using a calcium phosphate method. Zeocin resistant clones were transferred to a 96-well plate and assayed for IDEC-C2B8 production using an ELISA assay specific for the human constant region (4). Three IDEC-C2B8 samples were obtained from parallel cultures of a selected clone (CHO-tet-GnTIII-C2B8), differing only in the tetracycline concentration added to the medium (25, 50 and 2000 ng/mL respectively). Culture supernatants were harvested in the late exponential phase. An additional antibody sample was obtained from a CHO-tTA-derived clone, CHO-tTA-C2B8, cultured under identical conditions but without adding tetracycline to the medium. Antibody samples were purified from culture medium by protein A affinity chromatography and buffer exchanged to PBS on a cation exchange column as previously described (Umana, P., *et al.*, *Nat Biotechnol.* 17:176-180 (1999)). Antibody concentration was measured using a fluorescence-based kit from Molecular Probes (Leiden, The Netherlands) with Rituximab used as standard.

[0087] *Indirect immunofluorescence.* CD20-positive cells (SB cells; ATCC deposit no. ATCC CCL120) and CD20-negative cells (HSB cells; ATCC deposit

no. ATCC CCL120.1) were each incubated for 1h with 2.5 µg/ml of CHO-tet-GnTIII-derived IDEC-C2B8 antibody in Hank's balanced salt solution (GibcoBRL, Basel, Switzerland) and 2% bovine serum albumin fraction V (Roche Diagnostics, Rotkreuz, Switzerland) (HBSSB). As a negative control HBSSB was used instead of C2B8 antibody. A FITC-conjugated, anti-human Fc polyclonal antibody was used as a secondary antibody (SIGMA, St. Louis) for all samples. Cells were examined using a Leica fluorescence microscope (Wetzlar, Germany).

[0088] *Oligosaccharide profiling by MALDI/TOF-MS.* Neutral, N-linked oligosaccharides were derived from C2B8 antibody samples, MabThera™ (European counterpart of Rituximab; kind gift from R. Stahel, Universitätsspital, Switzerland), C2B8-25t, C2B8-50t, C2B8-2000t, and C2B8-nt, (100 µg each) as previously described (Umana, P., *et al.*, *Nat Biotechnol.* 17:176-180 (1999)). Briefly, the antibody samples were first treated with *Arthrobacter ureafaciens* sialidase (Oxford Glycosciences, Abingdon, UK) to remove any sialic acid monosaccharide residues. Neutral N-linked oligosaccharides were then released from the desialylated antibody samples using peptide-N-glycosidase F (Oxford Glycosciences), purified using micro-columns, and analyzed by MALDI/TOF-MS in an Elite Voyager 400 spectrometer (Perseptive Biosystems, Farmingham, MA).

[0089] *ADCC Activity Assay.* Peripheral blood mononuclear cells (PBMC) were separated from heparinated fresh human blood (in all experiments obtained from the same healthy donor) by centrifugation over a Ficoll-Paque (Pharmacia Biotech, Dübendorf, Switzerland) gradient. PBMC (effector) were depleted of monocytes by plastic adherence. CD20-positive SB (target) cells, were labeled for 90min with 100µCi <sup>51</sup>Cr (Amersham, Dübendorf, Switzerland) at 37 °C, washed twice in RPMI (GibcoBRL, Basel, Switzerland) and resuspended at a concentration of 10<sup>5</sup> cells/ml. Fifty microliters of C2B8 mAb diluted in RPMI medium was added to 100 µl SB cells (10,000 cells/well) in a 96-well round bottom microtiter plate (Greiner, Langenthal, Switzerland), centrifuged at 50xg

for 1min, and incubated for 1h at 4 °C. Subsequently, 50 µl of effector cell (suspended at  $2 \times 10^7$  cells/ml in RPMI medium) were added to each 96-well yielding a final E:T ratio of 100. Plates were incubated for 4h at 37 °C and 5%CO<sub>2</sub>, supernatant was harvested with a Skatron harvesting system (Skatron Instruments, Sterling, VA) and counted (ER, experimental release) in a Cobra 05005 γ counter (Canberra Packard, Meriden, CT). Maximum (MR) and spontaneous (SR) releases were obtained by adding, instead of C2B8 mAb, 100 µl of 1% Nonidet (Sigma, St. Louis) or 100µl of RPMI medium, respectively, to 100 µl labeled target cells. All data points were performed in triplicate. Specific lysis (%) was calculated with the following formula:  $(ER-SR)/(MR-SR) \times 100$ .

## Results and Discussion

[0090] *Production of IDEC-C2B8 and verification of specific antigen binding.* CHO-tet-GnTIII cells, with stable, tetracycline-regulated expression of GnTIII and stable, constitutive expression of IDEC-C2B8, were established and scaled-up for production of a set of antibody samples. During scale-up, parallel cultures from the same clone were grown under three different tetracycline concentrations, 25, 50 and 2000 ng/ml. These levels of tetracycline had previously been shown to result in different levels of GnTIII and bisected oligosaccharides (Umana, P., *et al.*, *Nat Biotechnol.* 17:176-180 (1999); Umana, P., *et al.*, *Biotechnol Bioeng.* 65:542-549 (1999)). A C2B8-producing, control cell line that does not express GnTIII was also established and cultured under the same conditions as for the three parallel cultures of CHO-tet-GnTIII. After Protein A-affinity chromatography, mAb purity was estimated to be higher than 95% by SDS-PAGE and Coomassie-blue staining. The samples were named according to the tetracycline concentration added to the culture medium for their production: C2B8-25t, C2B8-50t, C2B8-2000t and C2B8-nt (i.e., no tetracycline for the non-bisected control). Sample C2B8-25t showed specific antigen binding

by indirect immunofluorescence using CD20-positive and CD20-negative cells (FIG. 1), indicating that the synthesized VL and VH gene fragments were functionally correct.

[0091] *Oligosaccharide profiling with MALDI/TOF-MS.* The glycosylation profile of each antibody sample was analyzed by MALDI/TOF-MS of the released, neutral oligosaccharide mix. In this technique, oligosaccharides of different mass appear as separate peaks in the spectrum and their proportions are quantitatively reflected by the relative peak heights (Harvey, D. J., *Rapid Commun Mass Spectrom.* 7:614-619 (1993); Harvey, D. J., *et al.*, *Glycoconj J.* 15:333-338 (1998)). Oligosaccharide structures were assigned to different peaks based on their expected molecular masses, previous structural data for oligosaccharides derived from IgG1 mAbs produced in the same host, and information on the N-linked oligosaccharide biosynthetic pathway.

[0092] A clear correlation was found between GnTIII expression levels (i.e., tetracycline concentration) and the amount of bisected oligosaccharides derived from the different antibody samples. As expected, MabThera™ and C2B8-nt, which are derived from hosts that do not express GnTIII, did not carry bisected oligosaccharides (FIGS. 2A and 2B). In contrast, bisected structures amounted up to approximately 35% of the oligosaccharides pool in sample C2B8-2000t, i.e., at a basal level of GnTIII expression. In this case, the main bisected oligosaccharide peaks were of complex type, unequivocally assigned to peaks at  $m/z$  1689 and  $m/z$  1851 (FIG. 2C). The next higher GnTIII expression level, sample C2B8-50t, led to an increase in these peaks (including their associated potassium adducts at  $m/z$  1705 and 1861) of around 20%. This increase was accompanied by a concomitant reduction of their non-bisected counterparts at  $m/z$  1486 and 1648, respectively (FIG. 2D). At the highest GnTIII expression level, sample C2B8-25t, the main substrate for GnTIII,  $m/z$  1486, decreased to almost base-line level, while complex bisected structures ( $m/z$  1689 and 1851) decreased in favor of increases in peaks at  $m/z$  1664, 1810 and 1826 (FIG. 2E). These peaks can be assigned either to bisected hybrid compounds, to

galactosylated complex oligosaccharides, or to a mixture of both. Their relative increase, however, is consistent with the accumulation of bisected hybrid compounds, as GnTIII overexpression can divert the biosynthetic flux at early stages of the pathway (see FIG. 3A and 3B). The amount of bisected oligosaccharide structures (complex and hybrid type) reached approximately 80% for this sample.

[0093] *ADCC activity of IDEC-C2B8 glycosylated variants.* Different C2B8 mAb glycosylation variants were compared for ADCC activity, measured as in vitro lysis of CD20-positive SB cells. An additional mAb sample, C2B8-nt, derived from the parental cell line lacking GnTIII, was also studied. Sample C2B8-2000t produced at the basal GnTIII expression level and carrying low levels of bisected oligosaccharides was slightly more active than C2B8-nt (FIG. 4A). At the next higher level of GnTIII -expression, sample C2B8-50t carried approximately equal levels of bisected and non-bisected oligosaccharides, but did not mediate significantly higher target-cell lysis. However, at the lowest tetracycline concentration, sample C2B8-25t, which contained up to 80% of bisected oligosaccharide structures, was significantly more active than the rest of the samples in the whole antibody concentration range. It reached the maximal level of ADCC activity of sample C2B8-nt at a 10-fold lower antibody concentration (FIG. 4A). Sample C2B8-25t also showed a significant increase in the maximal ADCC activity with respect to the control (50% vs. 30% lysis).

[0094] Samples C2B8-50t and C2B8-25t, bearing the highest proportions of bisected oligosaccharides, were further compared in ADCC activity to Mabthera™, the version of Rituxan™ currently marketed in Europe (FIG. 4B). Sample C2B8-50t showed a slight increase in activity whereas sample C2B8-25t clearly outperformed Mabthera™ at all antibody concentrations. Approximately a five to ten-fold lower concentration of C2B8-25t was required to reach the maximal ADCC activity of Mabthera™, and the maximal activity of C2B8-25t was about 25% higher than that of Mabthera™.



[0095] These results show that, in general, the *in vitro* ADCC activity of the C2B8 antibody correlates with the proportion of molecules carrying bisected oligosaccharides in the Fc region. We had previously reported that in the case of chCE7, an antibody with a low baseline level of ADCC activity, significant increases of activity could be obtained by increasing the fraction of bisected oligosaccharides above the levels found in naturally-occurring antibodies (Umana, P., *et al.*, *Nat Biotechnol.* 17:176-180 (1999)). The same is true for the C2B8 mAb, which already has high ADCC activity in the absence of bisected oligosaccharides. In the case of chCE7, however, very large increases of ADCC activity were observed at a level of GnTIII expression where bisected oligosaccharides were predominantly of complex type (Umana, P., *et al.*, *Nat Biotechnol.* 17:176-180 (1999)). For the potent C2B8 mAb, such a large boost in activity was only observed at the highest levels of GnTIII expression studied, where bisected oligosaccharides had shifted mainly to the hybrid type (FIG. 2). For both mAbs, the samples with the highest activities had considerably higher levels of bisected than non-bisected oligosaccharides. Together, these observations indicate that probably both complex and hybrid bisected oligosaccharides are important for ADCC activity.

[0096] In both complex and hybrid oligosaccharides, a bisecting GlcNAc leads to a large change in oligosaccharide conformations (Balaji, P. V., *et al.*, *Int. J. Biol. Macromol.* 18:101-114 (1996)). The change occurs in a part of the oligosaccharide that interacts extensively with the polypeptide in the CH2 domain (Jefferis, R., *et al.*, *Immunol Rev.* 163:59-76 (1998)). Since the polypeptide is relatively flexible at this location (Jefferis, R., *et al.*, *Immunol Rev.* 163:59-76 (1998)), it is possible that the bisecting N-acetylglucosamine is mediating its biological effects through a conformational change in the Fc region. The potentially altered conformations would already exist in nature, as all serum IgGs carry bisected oligosaccharides. The main difference between the engineered and natural antibodies would be the proportion of molecules displaying the more active conformations.

[0097] Various approaches for increasing the activity of unconjugated mAbs are currently under clinical evaluation, including radio-immunotherapy, antibody-dependent enzyme/prodrugtherapy, immunotoxins and adjuvant therapy with cytokines (Hjelm Skog, A., *et al.*, *Cancer Immunol Immunother.* 48:463-470 (1999); Blakey, D. C., *et al.*, *Cell Biophys.* 25:175-183 (1994); Wiseman, G. A., *et al.*, *Clin Cancer Res.* 5:3281s-3296s (1999); Hank, J. A., *et al.*, *Cancer Res.* 50:5234-5239 (1990)). These technologies can give large increases in activity, but they can also lead to significantly higher side effects, elevated production costs and complex logistics from production to administration to patients when compared to unconjugated mAbs. The technology presented here offers an alternative way to obtain increases in potency while maintaining a simple production process, and should be applicable to many unconjugated mAbs.

#### EXAMPLE 2.

New Versions of the Anti-Renal Cell Carcinoma Antibody chG250 Having Enhanced Antibody-Dependent Cellular Cytotoxicity Obtained by Glycosylation Engineering of a chG250 Producing Cell Line

##### 1. Cell culture

[0098] SP2/0 mouse myeloma cells producing chG250 chimeric mAb (wt-chG250-SP2/0 cells) were grown in standard cell culture medium supplemented with 1:100 (v/v) penicillin/streptomycin/antimycotic solution (SIGMA, Buchs, Switzerland). Cells were cultured at 37 °C in a 5% CO<sub>2</sub> humidified atmosphere in Tissue Culture Flasks. Medium was changed each 3-4 days. Cells were frozen in culture medium containing 10% DMSO.

## 2. Generation of SP2/0 cells with pGnTIII-puro expression

[0099] wt-chG250-SP2/0 myeloma cells were transfected by electroporation with a vector for constitutive expression of GnTIII operatively linked via an IRES to a puromycin resistance gene. 24 hours before electroporation culture medium was changed and cells were seeded at  $5 \times 10^5$  cells/ml. Seven million cells were centrifuged for 4 min at 1300 rpm at 4 °C. Cells were washed with 3 mL new medium and centrifuged again. Cells were resuspended in a volume of 0.3-0.5 ml of reaction mix, containing 1.25% (v/v) DMSO and 20-30 µg DNA in culture medium. The electroporation mix was then transferred to a 0.4 cm cuvette and pulsed at low voltage (250-300 V) and high capacitance (960 µF) using Gene Pulser from Bio Rad. After electroporation cells were quickly transferred to 6 mL 1.25% (v/v) DMSO culture medium in a T25 culture flask and incubated at 37°C. Stable integrants were selected by applying 2 µg/mL puromycin to the medium two days after electroporation. After 2-3 weeks a stable, puromycin-resistant mixed population was obtained. Single-cell derived clones were obtained via FACS and were subsequently expanded and maintained under puromycin selection.

## 3. Western Blot

[0100] Puromycin-resistant clones were screened for GnTIII expression by Western blotting. The Western blots clearly showed that clones 5H12, 4E6 and 4E8 were expressing the highest levels of GnTIII. 5G2 also showed a GnTIII band of middle intensity, whereas 2F1, 3D3 and 4G3 had the lowest band intensities, therefore expressing lower amounts of GnTIII (FIG. 5).

4. Production and purification of chG250 monoclonal antibody from seven GnTIII-expressing clones including wild type

[0101] Clones 2F1, 3D3, 4E6, 4E8, 4G3, 5G2, 5H12 and the wild type (wt-chG250-SP2/0 cells) were seeded at  $3 \times 10^5$  cells/mL in a total volume of 130 ml culture medium, and cultivated in single Triple-flasks. Cells used for seeding were all in full exponential growth phase, therefore cells were considered to be at the same growth state when the production batches started. Cells were cultivated for 4 days. Supernatants containing the antibody were collected in the late exponential growth phase to ensure reproducibility. The chG250 monoclonal antibody was purified in two chromatographic steps. Culture supernatants containing the chG250 monoclonal antibody derived from each batch were first purified using a HiTrap Protein A affinity chromatography. Protein A is highly specific for the human IgG F<sub>c</sub> region. Pooled samples from the protein A eluate were buffer exchanged to PBS by cation-exchange chromatography on a Resource S 1ml column (Amersham Pharmacia Biotech). Final purity was judged to be higher than 95% from SDS-staining and Coomassie blue staining (FIG. 6). The concentration of each sample was determined with a standard calibration curve using wild type antibody with known concentration.

5. Oligosaccharide profiling of mAb preparations derived from the seven clones expressing different GnTIII levels

[0102] Oligosaccharide profiles were obtained by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI/TOF-MS), which accurately provides the molecular masses of the different oligosaccharide structures. This technique allows a quantitative analysis of proportions between different oligosaccharide structures within a mixture. Neutral oligosaccharides appeared predominantly as  $[M + Na^+]$  ions, however sometimes they were accompanied by smaller  $[M + K^+]$  ions, leading to an increase in mass of  $m/z$  of 16. The percentage of the structure appearing as potassium ion adducts depends

on the content of the matrix and may thus vary between samples. A mixture of neutral N-linked oligosaccharides derived from each antibody preparation was analyzed using a 2,5-dehydrobenzoic acid (2,5-DHB) as matrix. Some of the peaks in the spectra were unequivocally assigned to specific oligosaccharide structures, because of known monosaccharide composition and unique mass. However, sometimes multiple structures could be assigned to a particular mass. MALDI enables the determination of the mass and cannot distinguish between isomers. Knowledge of the biosynthetic pathway and previous structural data enable, in most cases, the assignment of an oligosaccharide structure to a peak in the spectrum.

- [0103] Oligosaccharides derived from the mAb sample produced in wt-chG250-SP2/0 cell line, that does not express GnTIII, contained nonbisected biantennary complex ( $m/z$  1486) and mono- or di-galactosylated nonbisected biantennary complex structures (FIG. 7A), both  $\alpha(1,6)$ -fucosylated in the core region (peaks  $m/z$  1648 and 1810 respectively).
- [0104] Expression of GnTIII generated bisected  $F_c$ -associated oligosaccharide structures of two types: complex or hybrid. Complex bisected oligosaccharides were unequivocally assigned to peaks at  $m/z$  1543, 1689, 1705, 1851 and 1867 ( $[M + K^+]$  adduct). As expected, the increase in bisected oligosaccharides was accompanied by a concomitant reduction of peaks  $m/z$  1486 and 1648, that correspond to nonbisected complex oligosaccharides. For all samples derived from the GnTIII expressing clones, the main substrate of GnTIII ( $m/z$  1486) decreased dramatically. As expected, the percentage of the nonbisected complex oligosaccharide type, assigned to peak at  $m/z$  1648, had the lowest values for the clones expressing the highest GnTIII levels (clones 4E6, 4E8, 5G2 and 5H12). These two peaks decreased in favor of the accumulation of bisected complex and bisected hybrid type oligosaccharides (FIGS. 7A-7D and 8A-8D). The percentage of bisected complex oligosaccharides was higher for the samples derived from the clones expressing lower amounts of GnTIII. This is consistent with the fact that a higher GnTIII expression level probably shifts the

biosynthetic flux to bisected hybrid structures, thereby decreasing the relative proportions of complex and complex bisected compound. For bisected hybrid structures, two possible structures could sometimes be assigned to a single peak. Therefore, some assumptions were made in order to approximate the percentage of these structures over the total oligosaccharide pool. Peaks  $m/z$  1664, 1680, 1810 and 1826 can be assigned to either bisected hybrid type, to galactosylated complex oligosaccharides, or a mixture of them. Due to the fact that the wt-antibody preparation had a relatively low percentage of peak 1664, it was assumed that this peak, appearing in significant amounts in the antibody samples derived from the different clones, corresponded entirely to bisected hybrid structures (FIGS. 7A-7D and 8A-8D). However to assign a specific structure to peaks  $m/z$  1810 and 1826 further characterization has to be performed. In summary, by overexpression of GnTIII, bisected oligosaccharides structures were generated and their relative proportions correlated with GnTIII expression levels.

6. Measurement of antibody mediated cytotoxic activity by Calcein-AM retention

[0105] The Calcein-AM retention method of measuring cytotoxicity measures the dye fluorescence remaining in the cells after incubation with the antibody. Four million G250 antigen-positive cells (target) were labelled with 10  $\mu$ M Calcein-AM (Molecular Probes, Eugene, OR) in 1.8 mL RPMI-1640 cell culture medium (GIBCO BRL, Basel, Switzerland) supplemented with 10% fetal calf serum for 30 min at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. The cells were washed twice in culture medium and resuspended in 12 mL AIMV serum free medium (GIBCO BRL, Basel, Switzerland). Labelled cells were then transferred to U-bottom 96-wells (30,000 cells/well) and incubated in triplicate with different concentrations of antibody for 1 hour at 4°C. Peripheral blood mononuclear cells (PBMC) were separated from heparinated fresh human blood (in all experiments obtained from the same healthy donor) by centrifugation over

a Ficoll-Paque (Pharmacia Biotech, Dübendorf, Switzerland) gradient. PBMCs were added in triplicate wells in a 50  $\mu$ L volume, yielding an effector to target ratio (E:T ratio) of 25:1 and a final volume of 200  $\mu$ L. The 96-well plate was then incubated for 4 hours at 37°C in a 5% CO<sub>2</sub> atmosphere. Thereafter the 96-well plate was centrifuged at 700 x g for 5 min and the supernatants were discarded. The cell pellets were washed twice with Hank's balanced salt solution (HBSS) and lysed in 200  $\mu$ L 0.05M sodium borate, pH 9, 0.1% Triton X-100. Retention of the fluorescent dye in the target cells was measured with a FLUOstar microplate reader (BMG LabTechnologies, Offenburg, Germany). The specific lysis was calculated relative to a total lysis control, resulting from exposure of the target cells to saponin (200  $\mu$ g/mL in AIMV; SIGMA, Buchs, Switzerland) instead of exposure to antibody. Specific lysis (%) was calculated with the following formula:

$$\% \text{ Cytotoxicity} = \frac{F_{\text{med}} - F_{\text{exp}}}{F_{\text{med}} - F_{\text{det}}}$$

where  $F_{\text{med}}$  represents the fluorescence of target cells treated with medium alone and considers unspecific lysis by PMBCs,  $F_{\text{exp}}$  represents the fluorescence of cells treated with antibody and  $F_{\text{det}}$  represents the fluorescence of cells treated with saponin instead of antibody.

[0106] To determine the effect of modified glycosylation variants of chG250 on the in vitro ADCC activity, G250 antigen-positive target cells were cultured with PBMCs with and without chG250 antibody samples at different concentrations. The cytotoxicity of unmodified chG250 antibody derived from the wild type cell line was compared with two antibody preparations derived from two cell lines (3D3, 5H12) expressing intermediate and high GnTIII levels, respectively (see FIG. 5).

[0107] Unmodified chG250 antibody did not mediate significant ADCC activity over the entire concentration range used in the assay (the activity was not significantly different from background). Augmented ADCC activity (close to

20%, see FIG. 9) at 2  $\mu\text{g/mL}$  was observed with the antibody sample derived from clone 3D3, which expressed intermediate GnTIII levels. The cytotoxic activity of this antibody samples did not grow at higher antibody concentrations. As expected the antibody preparation derived from clone 5H12 showed a striking increase over samples 3D3 and unmodified antibody in its ability to mediate ADCC against target cells. The maximal ADCC activity of this antibody preparation was around 50% and was remarkable in mediating significant ADCC activity at 125-fold less concentrated when comparing with the unmodified control sample.

### EXAMPLE 3

#### Treatment of Immune-Mediated Thrombocytopenia in a Patient with Chronic Graft-Versus-Host Disease

[0108] Autoimmune thrombocytopenia in chronic graft-versus-host disease represents an instance of B-cell dysregulation leading to clinical disease. To treat immune-mediated thrombocytopenia in a subject with chronic graft-versus-host disease, an anti-CD20 chimeric monoclonal antibody prepared by the methods of the present invention and having increased ADCC is administered to the subject as described in Ratanatharathorn, V. *et al.*, *Ann. Intern. Med.* 133(4):275-79 (2000) (the entire contents of which is hereby incorporated by reference). Specifically, a weekly infusion of the antibody, 375  $\text{mg/m}^2$  is administered to the subject for 4 weeks. The antibody therapy produces a marked depletion of B cells in the peripheral blood and decreased levels of platelet-associated antibody.

### EXAMPLE 4

#### Treatment of Severe, Immune-Mediated, Pure Red Cell Aplasia and Hemolytic Anemia

[0109] Immune-mediated, acquired pure red cell aplasia (PRCA) is a rare disorder frequently associated with other autoimmune phenomena. To treat immune-mediated, acquired pure red cell aplasia in a subject, an anti-CD20



chimeric monoclonal antibody prepared by the methods of the present invention and having increased ADCC is administered to the subject as described in Zecca, M. *et al.*, *Blood* 12:3995-97 (1997) (the entire contents of which are hereby incorporated by reference). Specifically, a subject with PRCA and autoimmune hemolytic anemia is given two doses of antibody, 375 mg/m<sup>2</sup>, per week. After antibody therapy, substitutive treatment with intravenous immunoglobulin is initiated. This treatment produces a marked depletion of B cells and a significant rise in reticulocyte count accompanied by increased hemoglobin levels.

[0110] It will be clear that the invention may be practiced otherwise than as particularly described in the foregoing description and examples. Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, are within the scope of the appended claims.

[0111] The entire disclosure of all publications (including patents, patent applications, journal articles, laboratory manuals, books, or other documents) cited herein are hereby incorporated by reference.

## WHAT IS CLAIMED IS:

1. A host cell engineered to produce a polypeptide having increased Fc-mediated cellular cytotoxicity by expression of at least one nucleic acid encoding  $\beta(1,4)$ -N-acetylglucosaminyltransferase III (GnT III), wherein said polypeptide produced by said host cell is selected from the group consisting of a whole antibody molecule, an antibody fragment, and a fusion protein which includes a region equivalent to the Fc region of an immunoglobulin, and wherein said GnT III is expressed in an amount sufficient to increase the proportion of said polypeptides carrying bisected hybrid oligosaccharides or galactosylated complex oligosaccharides or mixtures thereof in the Fc region relative to polypeptides carrying bisected complex oligosaccharides in the Fc region.
2. The host cell of claim 1, wherein said polypeptide is IgG or a fragment thereof.
3. The host cell of claim 1, wherein said polypeptide is IgG1 or a fragment thereof.
4. The host cell of claim 1, wherein said polypeptide is a fusion protein that includes a region equivalent to the Fc region of a human IgG.
5. The host cell of claim 1, wherein a nucleic acid molecule comprising at least one gene encoding GnTIII has been introduced into said host cell.
6. The host cell of claim 1, wherein said host cell has been engineered such that an endogenous GnT III gene is activated.

7. The host cell of claim 6, wherein said endogenous GnTIII has been activated by insertion of a DNA element which increases gene expression into the host chromosome.

8. The host cell of claim 6, wherein said host cell has been selected to carry a mutation triggering expression of an endogenous GnTIII.

9. The host cell of claim 8, wherein said host cell is the CHO cell mutant lec 10.

10. The host cell of claim 1, wherein said host cell is a CHO cell, a BHK cell, a NS0 cell, a SP2/0 cell, a Y0 myeloma cell, a P3X63 mouse myeloma cell, a PER cell, a PER.C6 cell or a hybridoma cell.

11. The host cell of claim 10, wherein said polypeptide is an anti-CD20 antibody.

12. The host cell of claim 11, wherein said anti-CD20 antibody is IDEC-C2B8.

13. The host cell of claim 10, wherein said host cell is a SP2/0 cell.

14. The host cell of claim 13, wherein said antibody is the chimeric anti-human renal cell carcinoma monoclonal antibody chG250.

15. The host cell of claim 5, wherein said at least one gene encoding GnTIII has been introduced into said host cell chromosome.

16. The host cell of claim 6, wherein said endogenous GnTIII has been activated by insertion of a promoter element, a transposon, or a retroviral element into the host cell chromosome.

17. The host cell of claim 1, further comprising at least one transfected nucleic acid encoding an antibody molecule, an antibody fragment, or a fusion protein that includes a region equivalent to the Fc region of an immunoglobulin.

18. The host cell of claim 1, wherein said at least one nucleic acid encoding a GnTIII is operably linked to a constitutive promoter element.

19. The host cell of claim 17, wherein said host cell comprises at least one transfected nucleic acid encoding an anti-CD20 antibody, the chimeric anti-human neuroblastoma monoclonal antibody chCE7, the chimeric anti-human renal cell carcinoma monoclonal antibody chG250, the chimeric anti-human colon, lung, and breast carcinoma monoclonal antibody ING-1, the humanized anti-human 17-1A antigen monoclonal antibody 3622W94, the humanized anti-human colorectal tumor antibody A33, the anti-human melanoma antibody directed against GD3 ganglioside R24, the chimeric anti-human squamous-cell carcinoma monoclonal antibody SF-25, an anti-human EGFR antibody, an anti-human EGFRvIII antibody, an anti-human PSMA antibody, an anti-human PSCA antibody, an anti-human CD22 antibody, an anti-human CD30 antibody, an anti-human CD33 antibody, an anti-human CD38 antibody, an anti-human CD40 antibody, an anti-human CD45 antibody, an anti-human CD52 antibody, an anti-human CD138 antibody, an anti-human HLA-DR variant antibody, an anti-human EpCAM antibody, an anti-human CEA antibody, an anti-human MUC1 antibody, an anti-human MUC1 core protein antibody, an anti-human aberrantly glycosylated MUC1 antibody, an antibody against human fibronectin variants containing the ED-B domain, or an anti-human HER2/neu antibody.

20. A method for producing a polypeptide in a host cell comprising culturing the host cell of any one of claims 1-19 under conditions which permit the production of said polypeptide having increased Fc-mediated cellular cytotoxicity.

21. The method of claim 20, further comprising isolating said polypeptide having increased Fc-mediated cellular cytotoxicity.

22. The method of claim 20, wherein said host cell comprises at least one nucleic acid encoding a fusion protein comprising a region equivalent to a Fc region of an immunoglobulin.

23. The method of claim 20, wherein greater than 50% of the oligosaccharides in the Fc region of said polypeptides are bisected.

24. The method of claim 20, wherein greater than 70% of the oligosaccharides in the Fc region of said polypeptides are bisected.

25. The method of claim 20, wherein the proportion of bisected hybrid oligosaccharides or galactosylated complex oligosaccharides or mixtures thereof in the Fc region is greater than the proportion of bisected complex oligosaccharides in the Fc region of said polypeptides.

26. The method of claim 20, wherein said polypeptide is the anti-CD20 antibody IDEC-C2B8 and the IDEC-C2B8 antibodies produced by said host cell have a glycosylation profile, as analyzed by MALDI/TOF-MS, that is substantially equivalent to that shown in FIG 2E.

27. The method of claim 20, wherein said polypeptide is the chG250 monoclonal antibody and the chG250 antibodies produced by said host cell have

a glycosylation profile, as analyzed by MALDI/TOF-MS, that is substantially equivalent to that shown in FIG 7D.

28. An antibody having increased antibody dependent cellular cytotoxicity (ADCC) produced by the method of claim 21.
29. The antibody of claim 28, wherein said antibody is selected from the group consisting of IDEC-C2B8, chCE7, ch-G250, a humanized anti-HER2 monoclonal antibody, ING-1, 3622W94, SF-25, A33, and R24.
30. An antibody fragment that includes a region equivalent to the Fc region of an immunoglobulin, having increased Fc-mediated cellular cytotoxicity produced by the method of claim 21.
31. A fusion protein that includes a region equivalent to the Fc region of an immunoglobulin and having increased Fc-mediated cellular cytotoxicity produced by the method of claim 21.
32. A pharmaceutical composition comprising the antibody of claim 28 and a pharmaceutically acceptable carrier.
33. A pharmaceutical composition comprising the antibody fragment of claim 30 and a pharmaceutically acceptable carrier.
34. A pharmaceutical composition comprising the fusion protein of claim 31 and a pharmaceutically acceptable carrier.
35. A method for the treatment of cancer comprising administering a therapeutically effective amount of the pharmaceutical composition of any one of claims 32-34 to a patient in need thereof.

36. An improved method for disease treatment based on B-cell depletion comprising administering a therapeutically effective amount of antibody to a human subject in need thereof, the improvement comprising administering a therapeutically effective amount of an antibody produced by the method of claim 28.

37. The improved method of claim 36, wherein said antibody is an anti-CD20 monoclonal antibody.

38. The improved method of claim 37, wherein said anti-CD20 antibody is IDEC-C2B8.

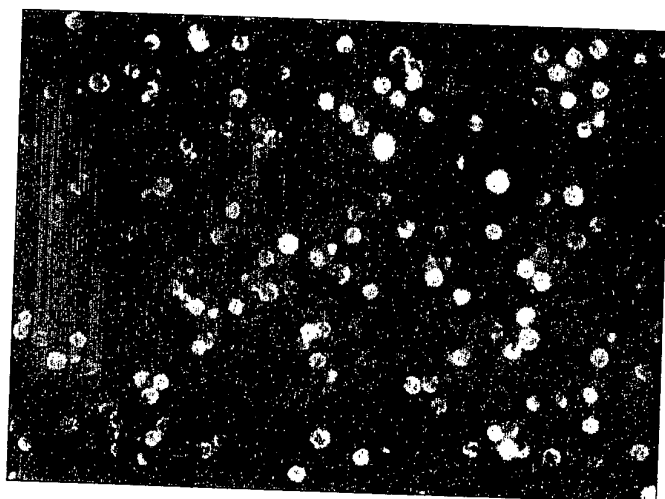


FIG. 1



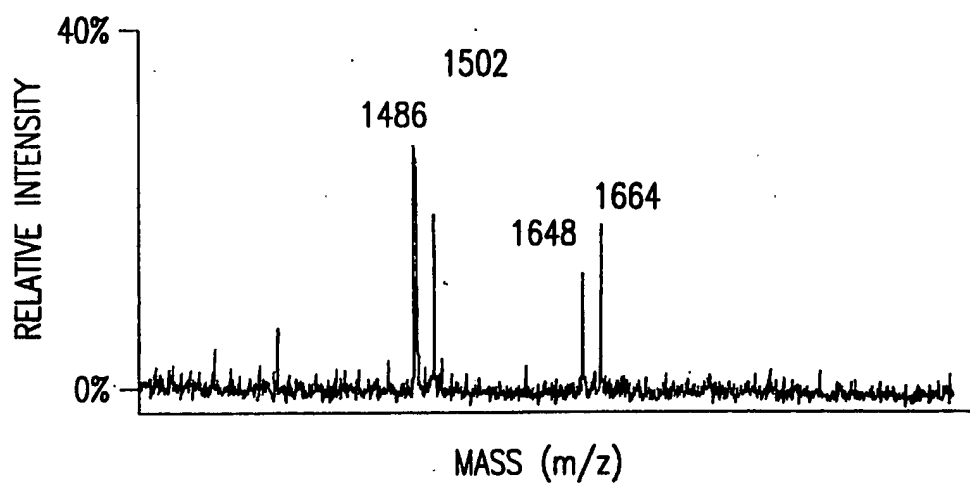


FIG. 2A

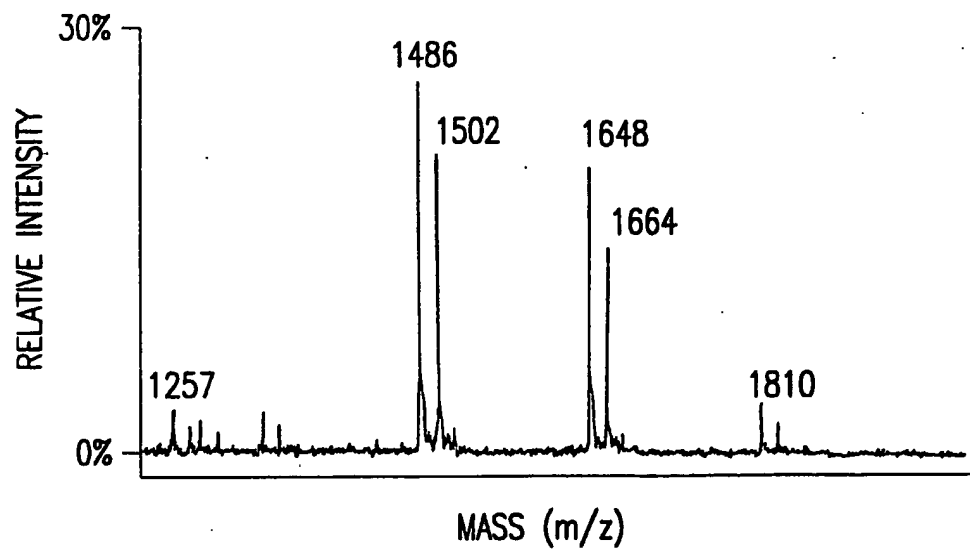


FIG. 2B

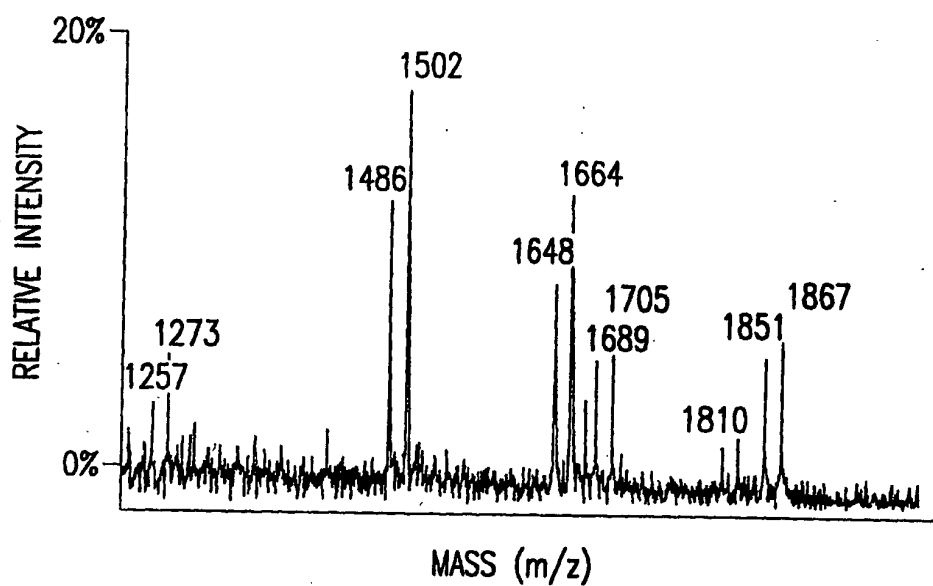


FIG.2C

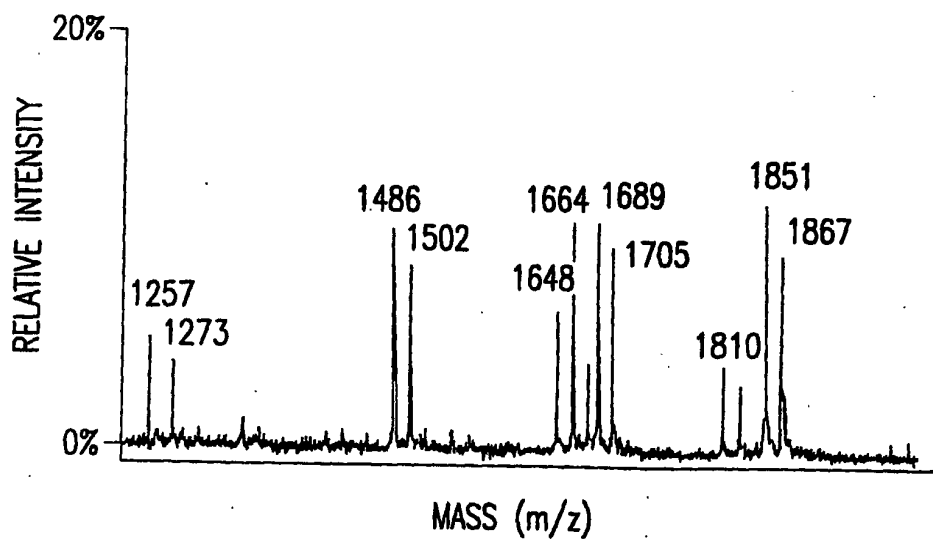


FIG.2D

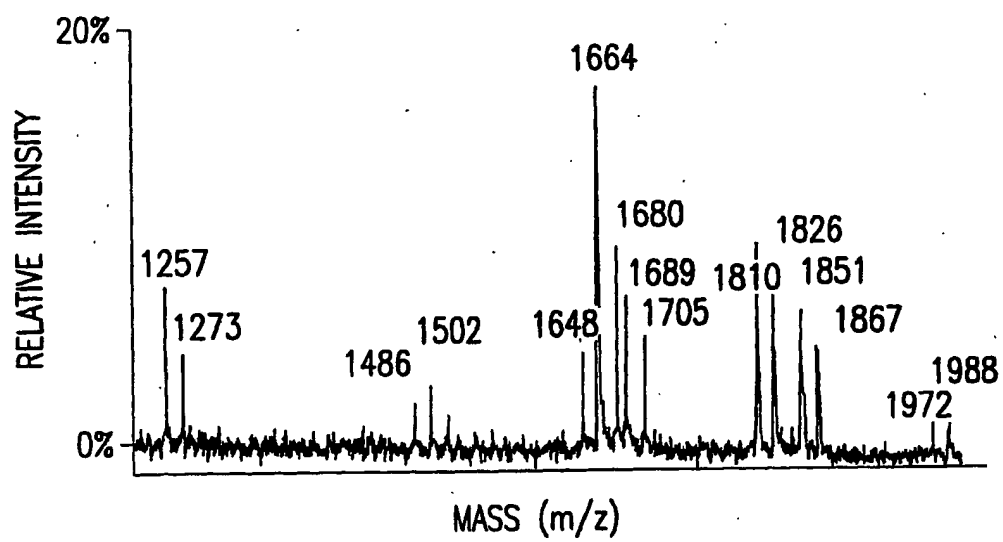


FIG.2E

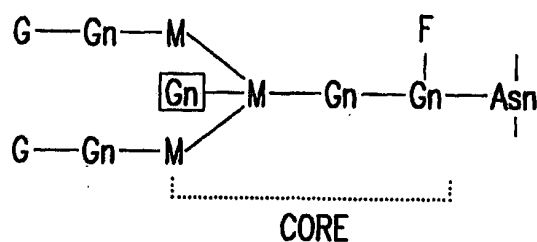


FIG.3A

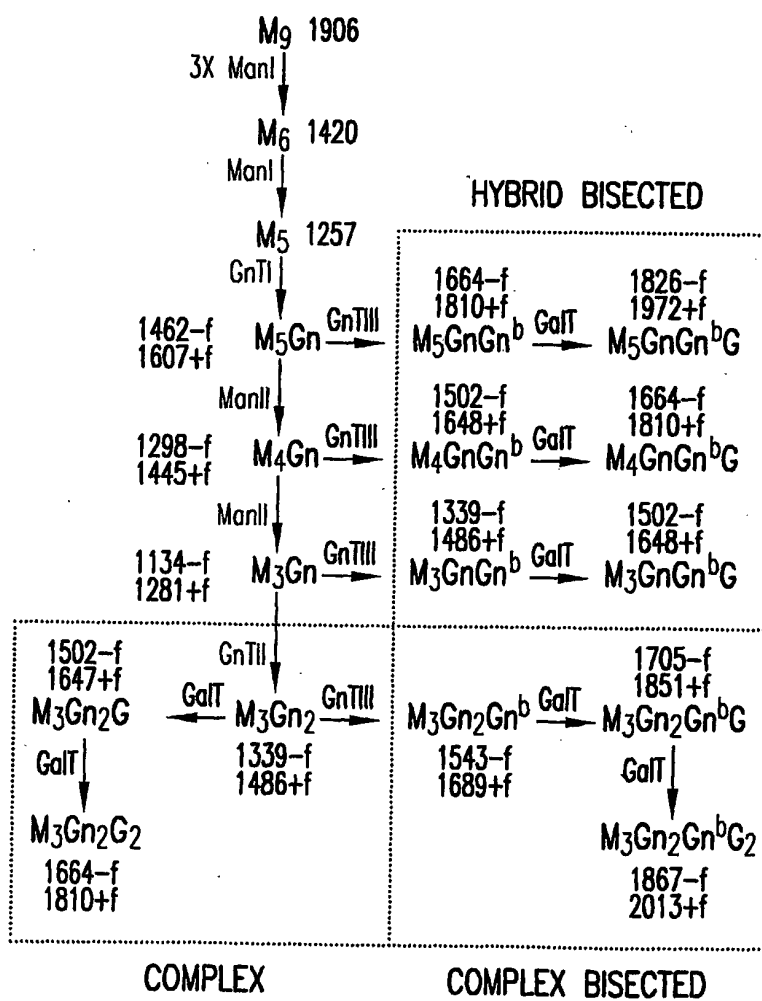


FIG.3B

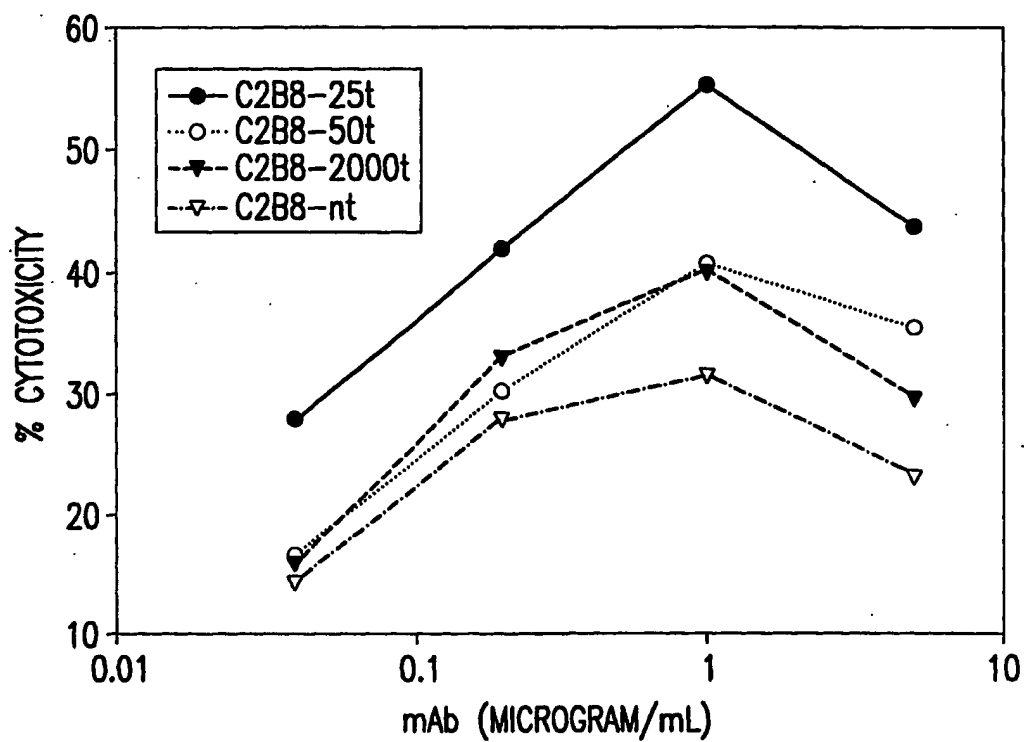


FIG. 4A

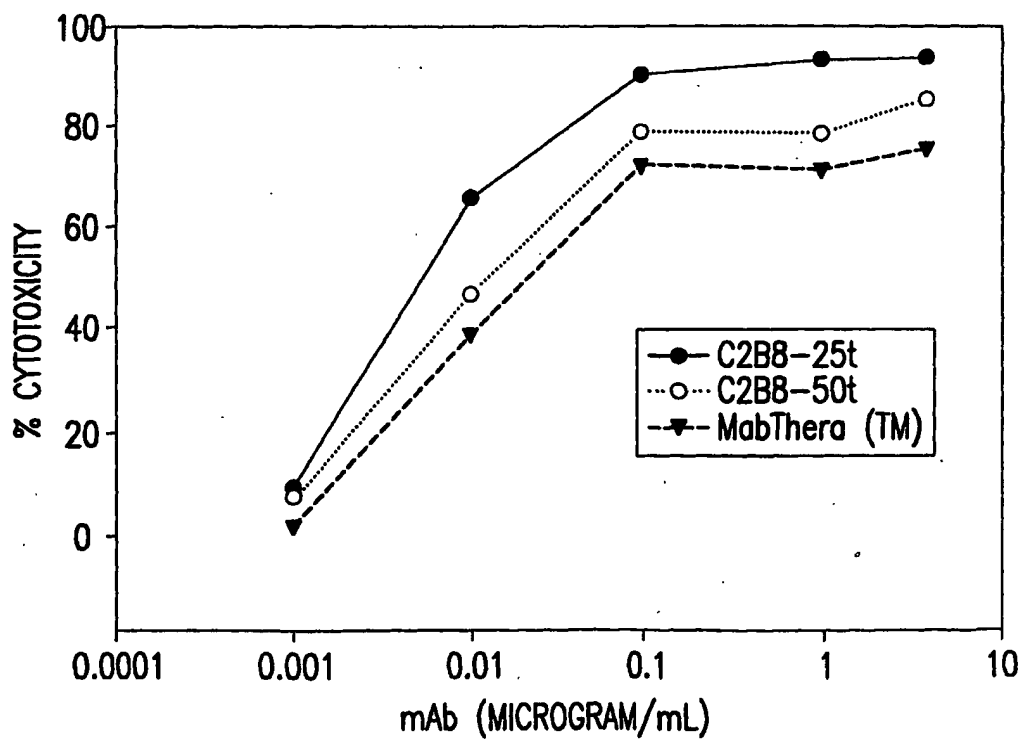
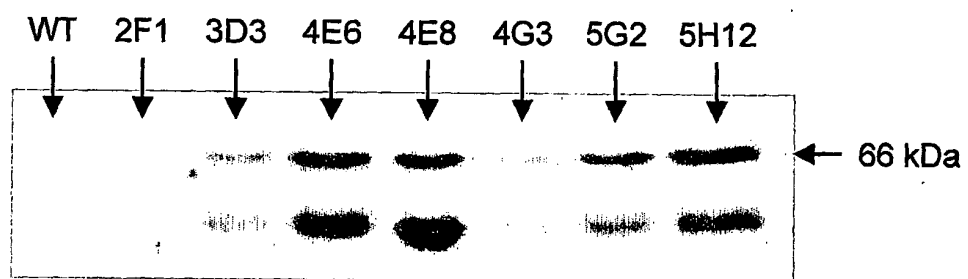


FIG. 4B

**FIG.5**

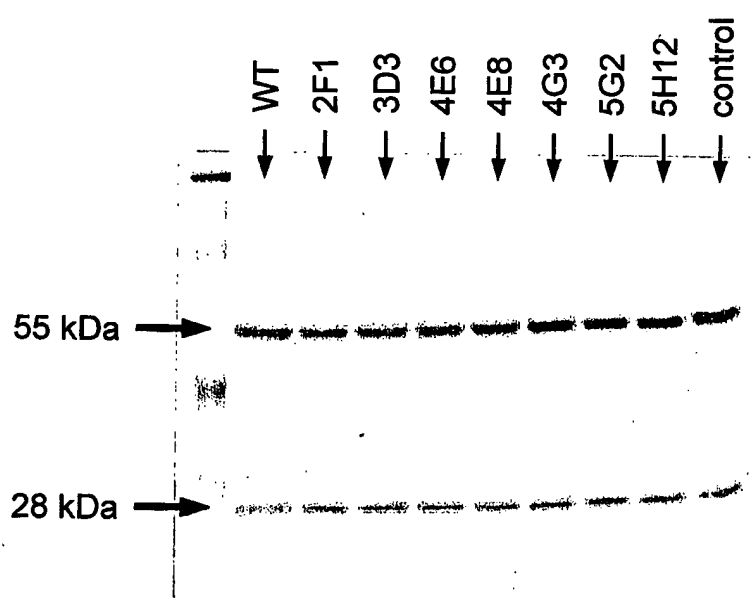
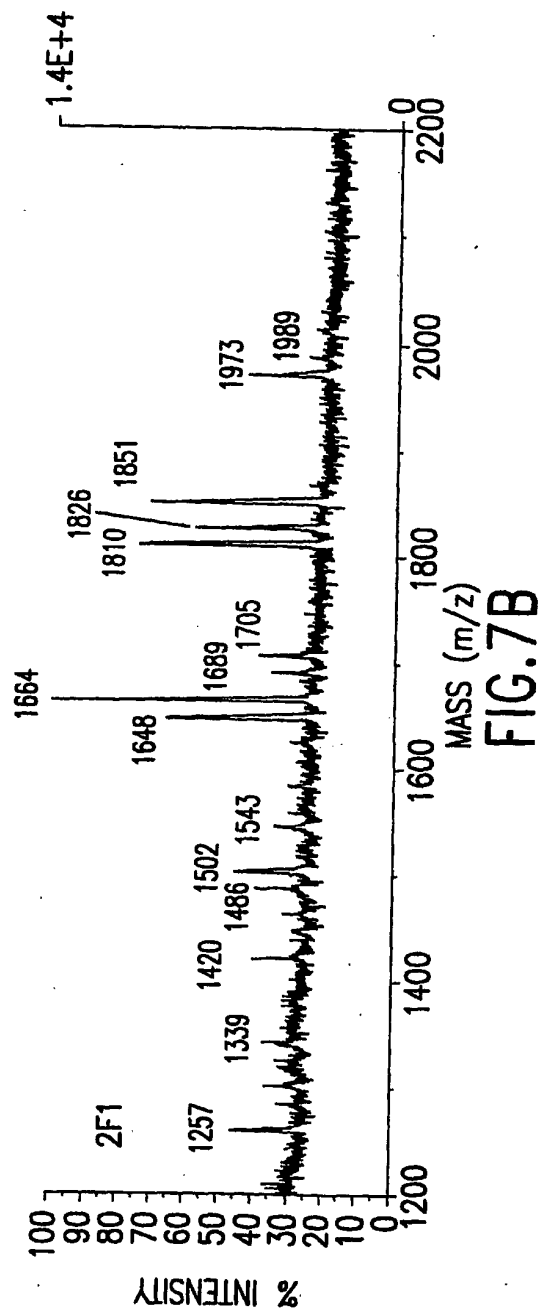
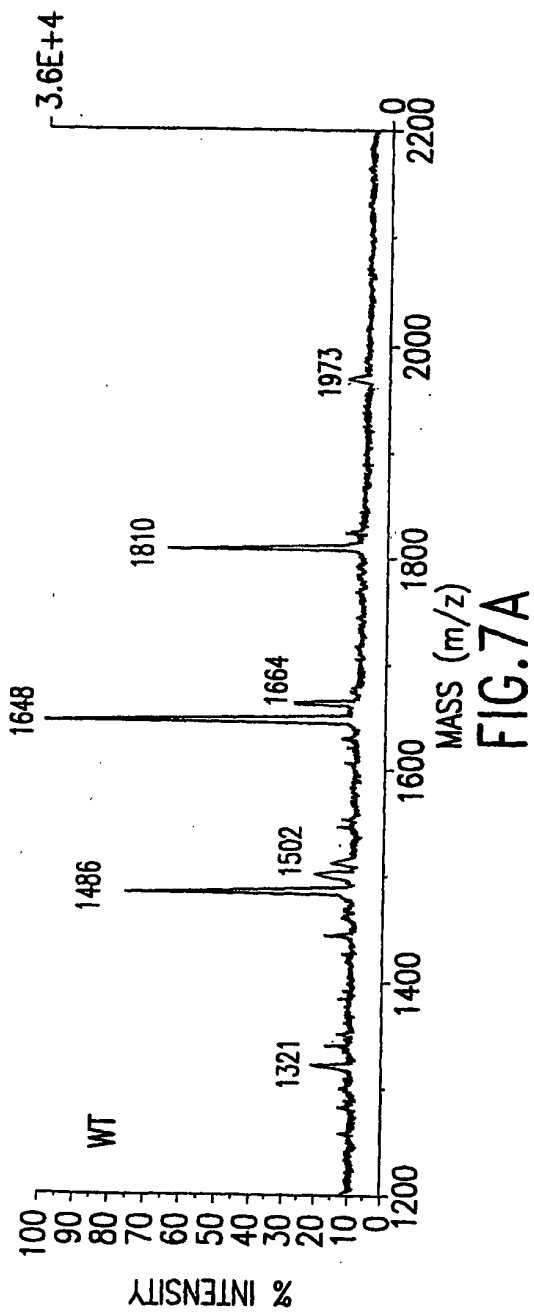
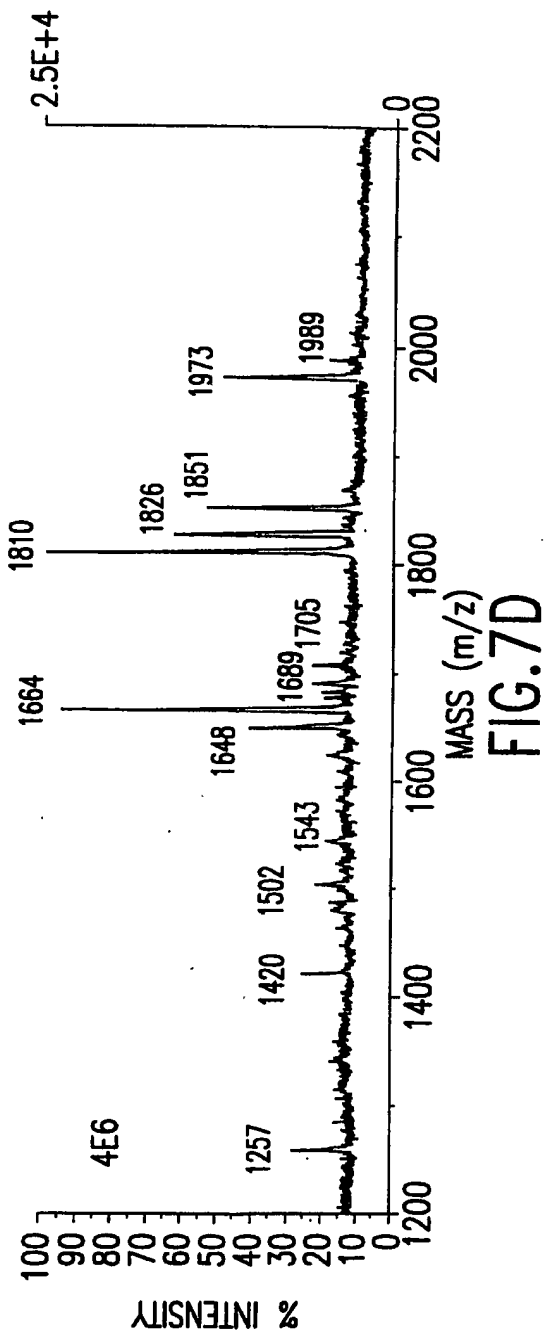
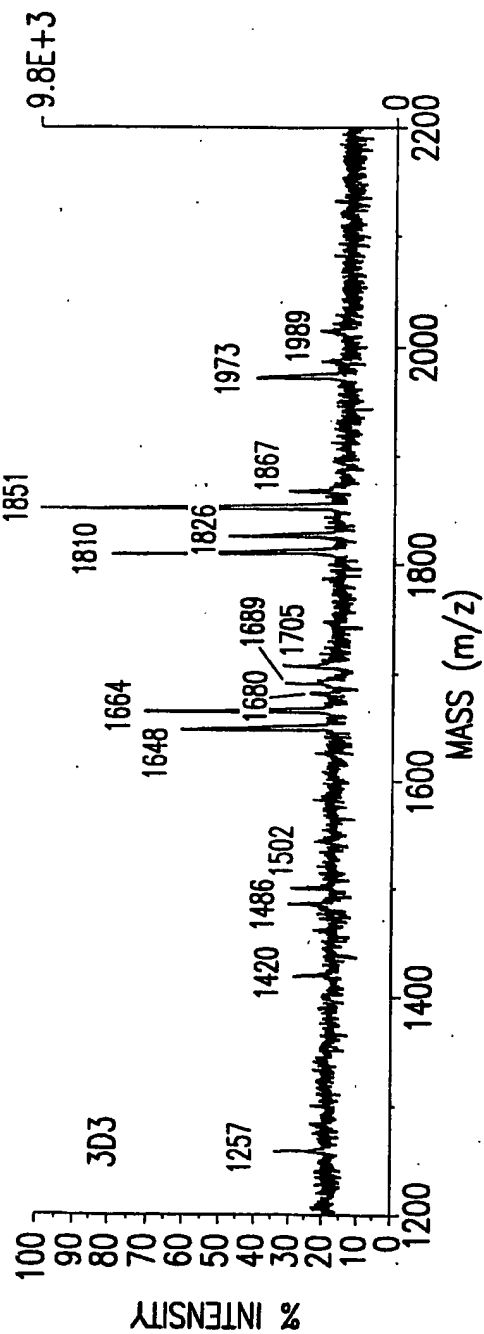
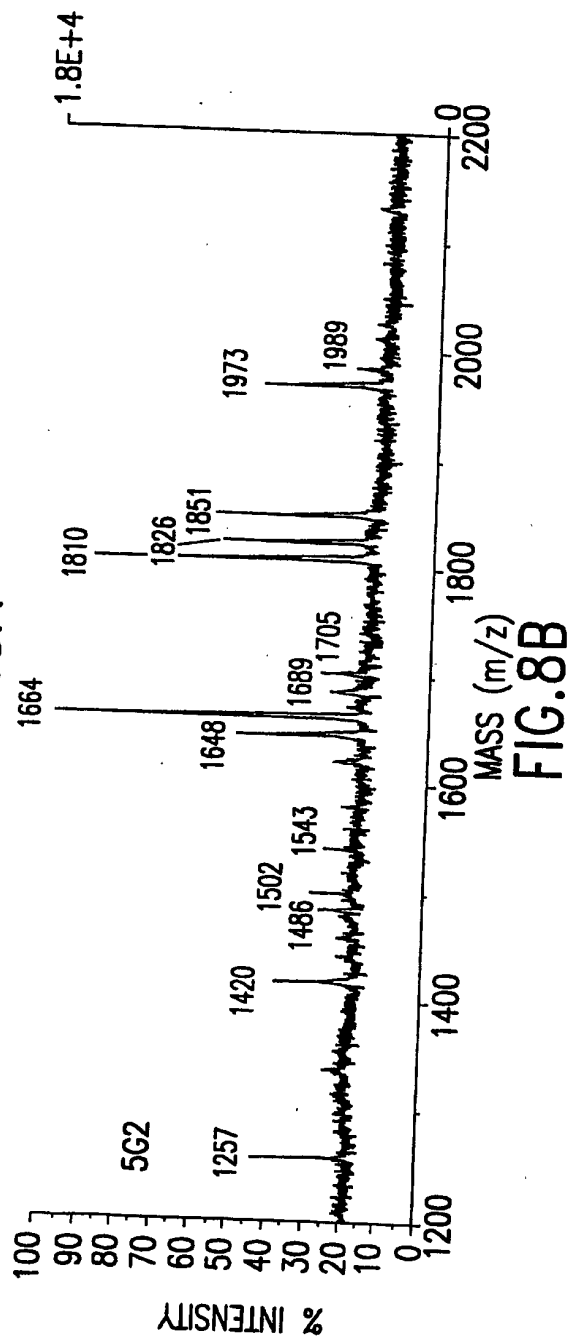
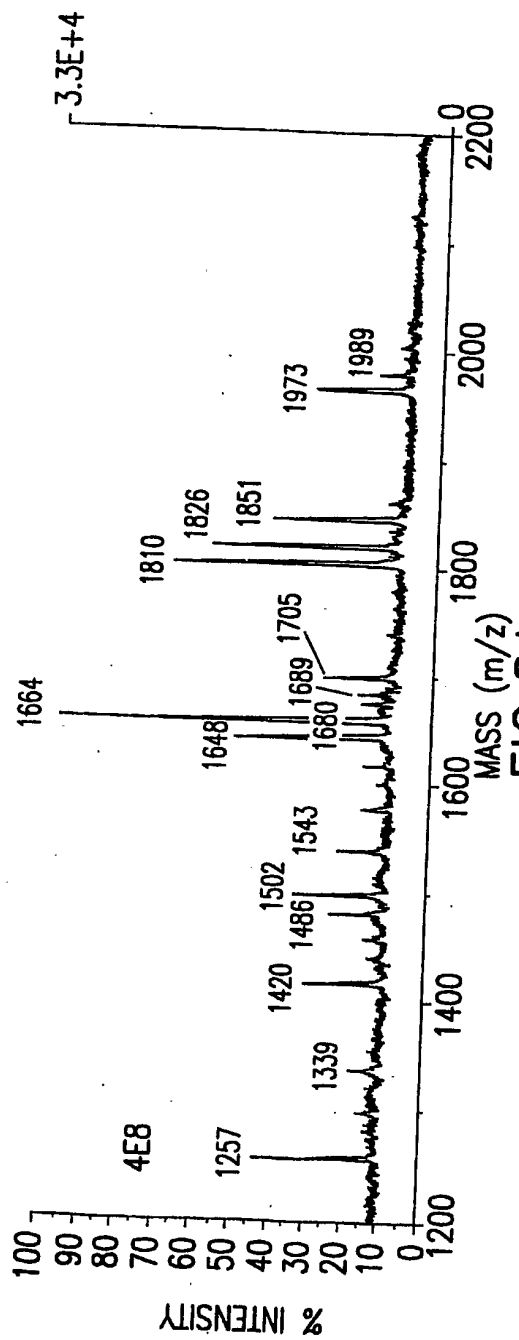


FIG.6









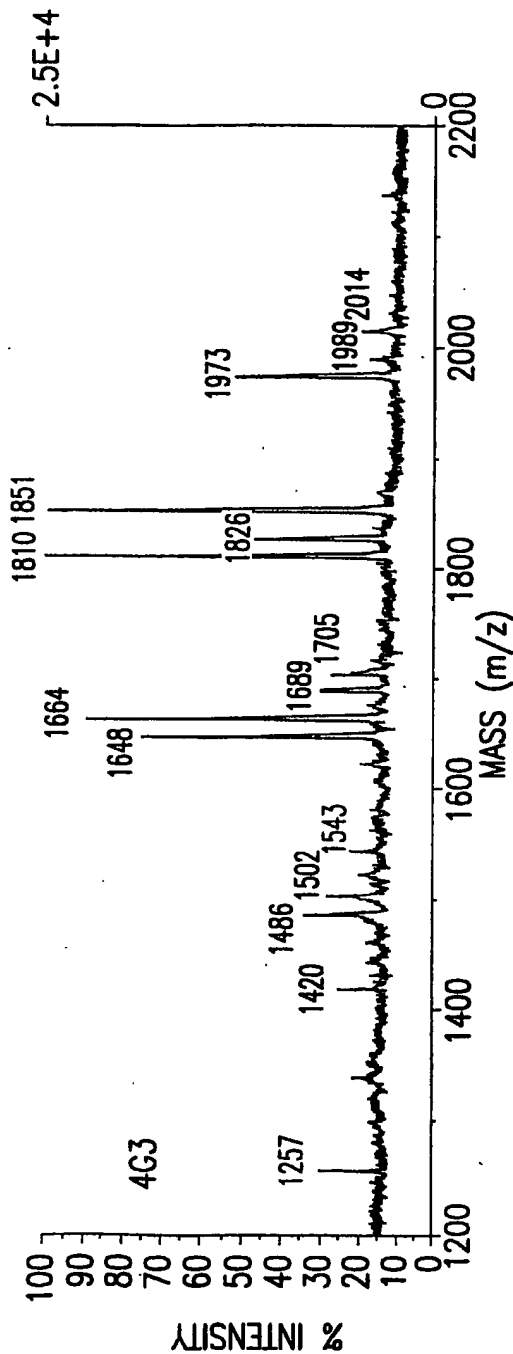


FIG. 8C

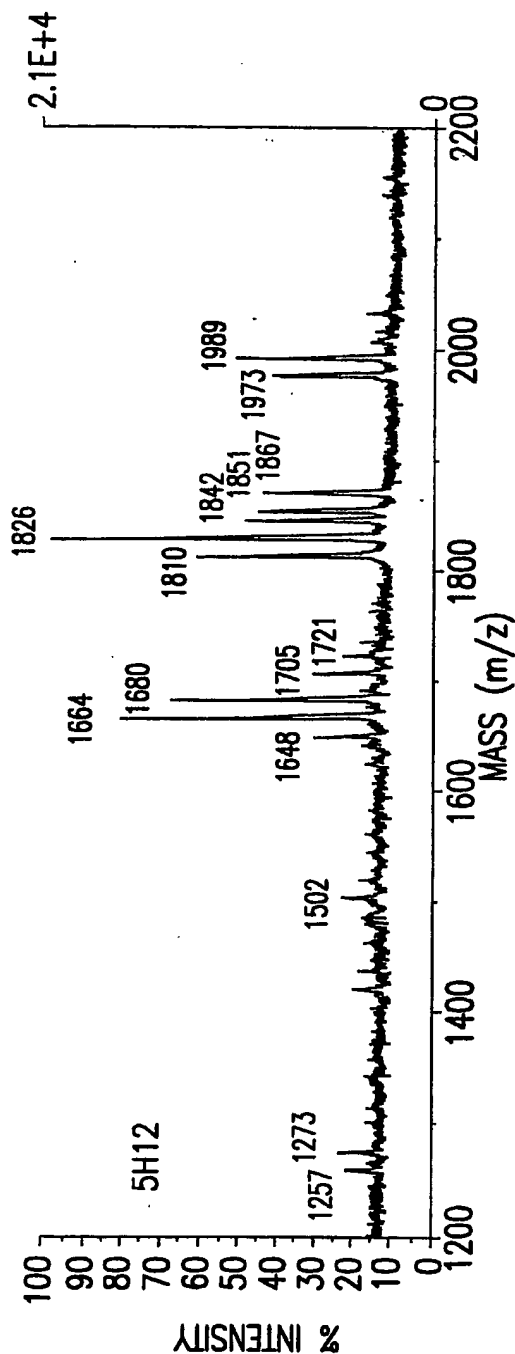


FIG. 8D

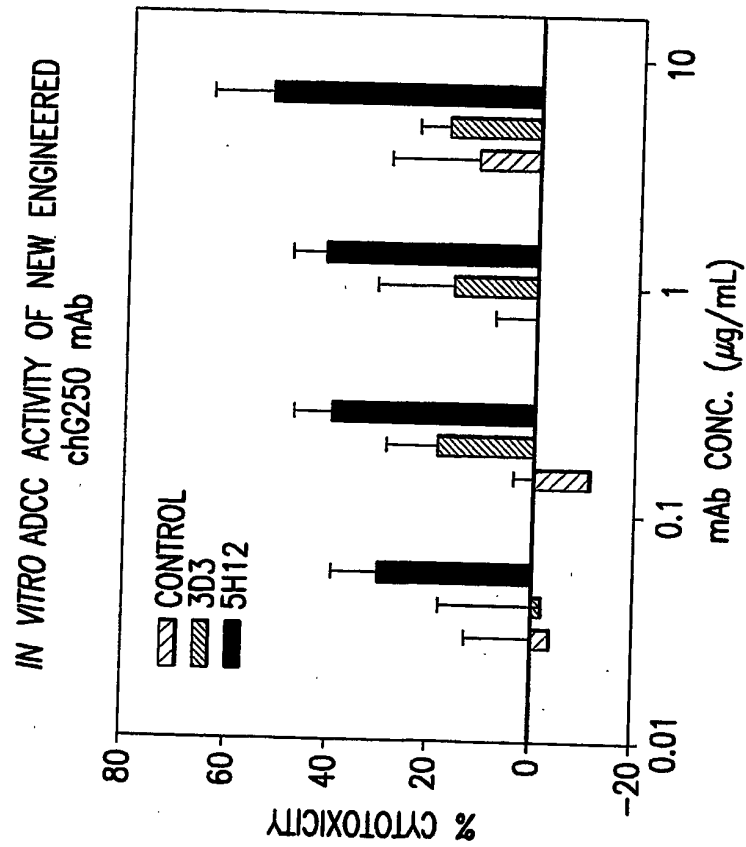


FIG. 9